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Mycoplasma hyorhinis disease model, proteomics, and vaccine efficacy

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Mycoplasma hyorhinis disease model, proteomics, and vaccine efficacy

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TABLE OF CONTENTS

LIST OF FIGURES v

LIST OF TABLES vi

LIST OF ABBREVIATIONS viii

ABSTRACT x

CHAPTER 1. INTRODUCTION 1
- General Introduction 1
- Dissertation Organization 1
- Literature Review 2
  - Porcine Mycoplasmas 2
  - *Mycoplasma hyorhinis* 4
    - Characteristics of disease 4
    - Phase shifting and antigenic variation 6
    - Pathogenesis and virulence factors 7
    - Immune response 9
  - Tissue culture 13
  - Cancer 14
- Summary with Hypothesis and Goals 15
- References 16

CHAPTER 2. DEVELOPMENT AND OPTIMIZATION OF A CELL-ASSOCIATED CHALLENGE MODEL FOR *MYCOPLASMA HYORHINIS* IN SEVEN-WEEK OLD CAESAREAN DERIVED-COLOSTRUM DEPRIVED PIGS 25
- Abstract 25
- Introduction 26
- Materials and Methods 27
  - Challenge material 27
  - Animal information 28
  - Experimental inoculation 29
  - Clinical observations 30
  - Gross pathological examination and sample collection 31
  - Histopathology 32
  - Real-time PCR 32
  - Bacteriology 34
  - Statistical analysis 34
- Results 37
  - Clinical observations 37
  - Gross pathology 40
  - Histopathology 42

PCR 44
Bacteriology 46
Discussion 47
Conclusion 50
Acknowledgements 50
References 51

CHAPTER 3. AGE SUSCEPTIBILITY OF CAESARIAN DERIVED COLOSTRUM DEPRIVED PIGS TO *MYCOPLASMA HYORHINIS* CHALLENGE 53
Abstract 53
Introduction 54
Materials and Methods 55
Challenge material 55
Animal information 55
Experimental inoculation 57
Clinical observations 57
Gross pathological examination and sample collection 58
Real-time PCR 59
Statistical analyses 59
Results 60
Clinical observations 60
Gross pathology 64
Real-time PCR 67
Discussion 69
Conclusion 72
References 73

CHAPTER 4. EFFICACY OF AN INACTIVATED *MYCOPLASMA HYORHINIS* VACCINE IN PIGS 74
Abstract 74
Introduction 75
Materials and Methods 76
Experimental design 76
Animals 76
Vaccine 77
Challenge 78
Observations 79
Sample collection 80
Statistical analysis 81
Results 82
Animals 82
Clinical observations 83
Gross pathology 85
Body weight 86

PCR 87
Discussion 88
Funding Information 90
Acknowledgments 90
References 90

CHAPTER 5. *MYCOPLASMA HYORHINIS* PROTEOMIC ANALYSIS: DIFFERENTIAL EXPRESSION BETWEEN *IN VITRO* AND *EX VIVO* CULTURES 92
Abstract 92
Background 92
Results 92
Conclusion 93
Background 93
Results 94
Identified proteins 94
Differential expression 96
Discussion 98
Conclusions 105
Materials and Methods 105
Sample preparation 105
Mass spectrometry 107
Identification of peptides 109
Comparative protein analysis 110
Author's Contributions 112
Acknowledgments 112
References 112

CHAPTER 6. CONCLUSION 116
General Discussion 116
Recommendations for Future Research 118
References 120

APPENDIX A. SUPPLEMENTAL INFORMATION FOR CHAPTER 2 123

APPENDIX B. SUPPLEMENTAL INFORMATION FOR CHAPTER 3 134

APPENDIX C. SUPPLEMENTAL INFORMATION FOR CHAPTER 4 139

APPENDIX D. SUPPLEMENTAL INFORMATION FOR CHAPTER 5 141

ACKNOWLEDGEMENTS 167

LIST OF FIGURES

Figure 2.1. Study 1 Clinical evaluations by group 38
Figure 2.2. Study 1 average daily weight gain by group 39
Figure 2.3. Study 2 clinical evaluations by group 39
Figure 2.4. Study 2 average daily weight gain by group 40
Figure 2.5. Study 2 assessment of arthritis by joint and group 42
Figure 2.6. Study 1 *in vitro* testing 44
Figure 2.7. Study 2 *M. hyorhinis* PCR for heart, lung, and joint swabs 46

Figure 3.1. Assessment of lameness 61
Figure 3.2. Average daily weight gain 63
Figure 3.3. Gross assessment for polyserositis 65
Figure 3.4. Assessment for arthritis 66
Figure 3.5. *M. hyorhinis* PCR for necropsy swabs 68

Figure 4.1. Clinical observations post-challenge 84
Figure 4.2. Duration of lameness 84
Figure 4.3. Pericarditis 85
Figure 4.4. Analysis of body weight 87

Figure 5.1. Histograms of protein coverage 95
Figure 5.2. Venn diagram for intersection of comparisons for differential expression 97
Figure 5.3. Heat map for distribution of proteins among the culture types which were significant (p<0.05) for differential expression 100

LIST OF TABLES

Table 2.1. List of Primers, Probes and Control Oligo Sequences 33
Table 2.2. Histopathology scores for Study 1 43

Table 3.1. Scoring system for lameness 58
Table 3.2. Mean weight (lbs) on first and last day of challenge 62

Table 4.1. Clinical observation scoring description 79

Table 5.1. Protein identification metrics 95

Table S1. Study 1 dose and route of administration on day of study 123
Table S2. Study 2 dose and route of administration of day of study 123
Table S3. Scoring system for respiration, coughing, and lameness 124
Table S4. Schedule of Events for Study 1 124
Table S5. Schedule of Events for Study 2 125
Table S6. Study 1 average daily gain (ADG) statistical summary 125
Table S7. Study 1 polyserositis statistical summary 126
Table S8. Study 1 arthritis statistical summary 127
Table S9. Study 1 PCR results statistical summary 128
Table S10. Study 1 lameness statistical summary 129
Table S11. Study 1 Histology statistical summary 130
Table S12. Study 2 average daily gain (ADG) statistical summary 131
Table S13. Study 2 polyserositis statistical summary 131
Table S14. Study 2 PCR results statistical summary 131
Table S15. Study 2 arthritis statistical summary 132
Table S16. Study 2 clinical observations statistical summary 133

Table S17. Challenge dose and route of administration on day of study 134
Table S18. Schedule of events 134
Table S19. Statistical summaries for weight measurements 135
Table S20. Statistical summaries for polyserositis 136

Table S21. Statistical summaries for *M. hyorhinis* PCR 137

Table S22. Statistical summaries for arthritis 138

Table S23. Statistical summary for lameness 138

Table S24. Summary of observations for abnormal respirations and coughing 139

Table S25. Summary of observations for lameness 139

Table S26. Summary of incidence of pericarditis 139

Table S27. Summary of weight gain in the vaccination phase and challenge phase 140

Table S28. Summary of average daily weight gain (ADG) 140

Table S29. Identification of 34 proteins unique to the Broth vs. McCoy comparison and significant ($p < 0.05$) for differential expression 141

Table S30. Identification of 20 proteins unique to the Broth vs. MDCK comparison and significant ($p < 0.05$) for differential expression 142

Table S31. Identification of 22 proteins unique to the MDCK vs. McCoy comparison and significant ($p < 0.05$) for differential expression 143

Table S32. Identification of overlapping proteins common to all three comparisons (Broth vs. McCoy, Broth vs. MDCK, and MDCK vs. McCoy) and significant ($p < 0.05$) for differential expression 144

Table S33. Identification of 29 overlapping proteins common to both Broth vs. *ex vivo* comparisons (Broth vs. McCoy and Broth vs. MDCK) and significant ($p < 0.05$) for differential expression 145

Table S34. McCoy culture *M. hyorhinis* proteins 146

Table S35. MDCK culture *M. hyorhinis* proteins 152

Table S36. Broth culture *M. hyorhinis* proteins 160

LIST OF ABBREVIATIONS

A – adenine

BIVI – Boehringer Ingelheim Vetmedica, Inc.

bp – base pair

C – cytosine

CDCD – caesarian-derived, colostrum deprived

CFR – Code of Federal Regulations

CI – confidence interval

Cq - Quantification cycle, threshold fluorescence value

Ct – crossing threshold

DMEM – Dulbecco's modified essential medium

DNA - Deoxyribonucleic acid

ELISA – enzyme-linked immunosorbent assay

EP – enzootic pneumonia

FDR – false discovery rate

G – guanine

IFNγ – gamma interferon

IgA – immunoblobulin alpha

IgG – immunoglobulin gamma

IL-1β – interleukin 1 beta

IL-6 – interleukin 6

IL-8 – interleukin 8

IL-10 – interleukin 10

IN – intranasal

iNOS – induced nitric oxide synthase

IP – intraperitoneal

IT – intratracheal

IV – intravenous

ISU – Iowa State University

kDa – kilo-Dalton

LC-MS/MS – liquid chromatography, tandem mass spectrometry

MDCK – Madin-Darby canine kidney cells

MEM – modified essential medium

MF – *Mycoplasma flocculare*

MHP – *Mycoplasma hyopneumoniae*

MHR – *Mycoplasma hyorhinis*

MHS – *Mycoplasma hyosynoviae*

mRNA – messenger RNA

NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells

NO – nitric oxide

nt – nucleotide

ORF – open reading frame

PCR – polymerase chain reaction

PRDC – porcine respiratory disease complex

PRRSv – porcine reproductive and respiratory syndrome virus

qPCR – relative quantitative PCR

RNA – Ribonucleic acid

rRNA – ribosomal ribonucleic acid

SE – standard error(s)

SEM – Spiroplasmatacae-Entomoplasmatacease

T – thymine

Th1 – T helper 1

Th2 – T helper 2

TLR – Toll-like receptor

TNFα – tumor necrosis factor alpha

TPP – thiamine pyrophosphate

µm – micro-meter

USDA – United States Department of Agriculture

Vlp – variable lipoprotein

ABSTRACT

The studies discussed herein were conducted to develop a vaccine for use in alleviating *Mycoplasma hyorhinis* associated disease in swine. This goal necessitated the development of a consistent and robust challenge model. We exploited *M. hyorhinis'* affinity to infect tissue culture and have generated a cell-associated challenge material. This material was then administered in a consecutive-day, variable route model resulting in a high degree of lameness, arthritis and polyserositis in caesarian derived, colostrum deprived pigs. We further demonstrated an age limitation of the pigs for susceptibility to *M. hyorhinis* infection in this model. An inactivated whole-cell vaccine was developed and efficacy was evaluated in the challenge model. Vaccination provided significant reductions of pericarditis, arthritis and lameness as well as a significant increase in weight gain compared to a placebo control group. Lastly, a comparative proteomic analysis was performed between a broth-derived culture and two cell-associated cultures of a single isolate of *M. hyorhinis*. Differential expression of several membrane-associated proteins was observed in the cell-associated cultures. The identified proteins provide insight into the infection process and are targets for future vaccine trials.

CHAPTER 1. INTRODUCTION

General Introduction

Mycoplasma hyorhinis (MHR) is a pathogen of swine which causes polyserositis and lameness in the production setting, leading to reduced performance or culling of affected animals. These outcomes result in economic losses to the pig producer. As compared with *Mycoplasma hyopneumoniae*, a major respiratory pathogen of swine, our understanding of the pathogenesis of MHR is poor. As no commercial vaccines are currently available, treatment of MHR-associated disease has been mainly through antibiotic therapy. The studies described here detail a disease model developed for use to evaluate vaccine candidates. A proteomic evaluation is also discussed which will aid in our understanding of MHR infection.

Dissertation Organization

This dissertation is arranged into six chapters. The first chapter is a general introduction to MHR and includes a literature review. Chapters 2 and 3 detail the development and optimization of a disease model for MHR, as well as the age susceptibility of pigs to this model. Chapter 4 determines the efficacy of an inactivated, whole cell vaccine for protection against MHR-associated pericarditis and lameness. Chapter 5 details a comparative proteomic analysis between *in vitro* and *ex vivo* cultures of a single isolate of MHR for differential protein expression. The final chapter is a general conclusion with discussions for future research. Chapters 2 - 5 constitute research which has or will be submitted for publication and are presented in the style of the peer reviewed journal for which they were targeted.

Literature Review

Porcine Mycoplasmas

Mycoplasmas are bacteria in the family *Mycoplasmataceae* and are in the class *Mollicutes*. This class of bacteria is distinguished by their small size (0.1 µm – 0.3 µm), very small genome with a low G + C content, and lack of a cell wall (1, 2). It is proposed that Mycoplasmas' small, reductive genome de-evolved from a common Gram positive ancestor, possibly lactobacilli, streptococci, or staphylococci (1-3).

Three distinct mycoplasma species are considered pathogenic to swine, *Mycoplasma hyopneumoniae* (MHP), *Mycoplasma hyosynoviae* (MHS), and *Mycoplasma hyorhinis* (MHR) (4, 5). Other species of Mycoplasma, such as *Mycoplasma flocculare* (MF), can be found in this host but have yet to be associated with disease (1, 5-7). The most well-known and researched swine mycoplasma is MHP, the etiologic agent of enzootic pneumonia (EP) (4, 8-10). Phylogenetic analysis of 16S rRNA gene sequences cluster most of the *Mycoplasma* spp. into the *Mycoplasmataceae* family; the exceptions being the *Mycoplasma mycoides* subgroup (including *Mycoplasma mycoides* mycoides and *Mycoplasma capricolum* capricolum), which cluster to the *Spiroplasmatacae-Entomoplasmatacease* (SEM) family (1). The *Mycoplasmataceae* phylogeny divides into α-, β-, γ- and δ-mycoplasma branches, of which MHS clusters to the β branch and MHR, MHP and MF to the γ branch (1).

The three pathogens (MHR, MHP, and MHS) share some culture requirements and disease characteristics. Both MHR and MHP ferment glucose, whereas MHS hydrolyses arginine for energy requirements (1, 5). However, all three require an exogenous source of sterols and fatty acids as they lack the machinery to synthesize these fundamental membrane components (1, 11). Likewise, they lack the capability of *de novo* synthesis of nucleic acid

precursors and amino acids which they must acquire from their environment. Supplementation of growth medium with yeast extract and animal serum generally fulfill these requirements (5, 11).

These three species of *Mycoplasma* all colonize the upper respiratory tract and can be recovered from the nasal cavity and tonsil of affected pigs (4, 12-14). Although MHP is the major agent for EP, MHR has also been shown to cause pneumonia and both species are frequently isolated together from the lungs of pigs (5, 15-18). Similarly, both MHR and MHS cause lameness and arthritis and can be isolated from the joint fluids and synovial membranes of affected pigs, though infrequently together (5, 14, 18-21). Further, MHR also causes polyserositis and can be isolated from serosal tissues (i.e. pericardium, pleura, and peritoneum) (5, 18, 19, 21).

Treatment of mycoplasma infections in pigs has largely depended on antibiotics. Due to the lack of a cell wall, β-lactams are ineffective. Macrolides, tetracyclines, and fluoroquinolones have proven effective in reducing clinical signs, although continuous or pulse dosing is required (5, 10). These dosing procedures are expensive, often do not affect overall bacterial population and increase the risk of development of antibiotic resistance (5, 10, 18, 22, 23). While whole-cell, inactivated commercial vaccines have been used to control MHP infections for several years, results of vaccination protocols have been mixed (5, 24-26). There are currently no commercially available vaccines for MHR or MHS. The efficacy of an experimental MHR vaccine is discussed in Chapter 5 of this dissertation.

Management of production practices, biosecurity and air flow (i.e. all-in/all-out, gilt exposure, positive-pressure barns, etc.) have also proven effective in control of MHP,

especially in combination with vaccination and antimicrobial protocols (26). These measures have not been fully investigated in mitigation of MHR and MHS.

Mycoplasma hyorhinis

Characteristics of disease

Mycoplasma hyorhinis is ubiquitous in the pig population and can be found in the upper respiratory tract of both healthy animals and those showing clinical signs of MHR infection (14, 18, 21). Clinical signs vary greatly from severe systemic infection leading to polyserositis, arthritis and lameness, to mild clinical manifestations such as otitis media or ocular discharge (5, 17-21, 27-34). In addition to the nasal cavity, ears, tonsils, lungs, joints, and serous membranes of the visceral organs, MHR has also been detected in the brains of some pigs, although no definitive association to neurological disease has been made (5, 18).

The gross lesions of MHR (serofibrinous to fibrinopurulent pericarditis, pleuritis, and peritonitis) are similar to, and are often confused with, those of the bacterial swine pathogens *Streptococcus suis* and *Haemophilus parasuis* (5, 21, 35). Both MHR and *H. parasuis* are often co-cultured from affected animals (35). Gross observations thus necessitate confirmatory testing (i.e. culture, polymerase chain reaction (PCR), etc.) for a definitive diagnosis. Histological examination of synovial and serosal membranes usually show invasion by polymorphonuclear and mononuclear cells, hyperplasia of the cells in the synovial lining, and villous hypertrophy of the synovial membrane (5, 19, 21, 27, 28). Macroscopic and microscopic lesions can be exacerbated by viral co-infections such as

porcine reproductive and respiratory syndrome virus (PRRSv) and porcine circovirus type 2 (PCV2), both members of the porcine respiratory disease complex (PRDC) (35, 36).

Pigs are reported to be most susceptible to MHR infection from weaning to approximately 10 weeks of age (5, 18, 21, 27, 37); whereas lameness associated with MHS tends towards animals older than approximately 12 weeks of age (5, 21, 37, 38). It is uncertain whether exposure of MHR in younger animals increases their susceptibility to MHS clinical disease later in life. It is suspected that piglets become exposed to MHR while on the sow and that variations in virulence between field strains of MHR, co-infections, genetic predisposition of the animals, and stress are triggers for MHR infections to progress from sub-clinical to clinical (18). Chronic arthritis has been observed in older animals affected with MHR (27, 30, 34). Age susceptibility to MHR infection is presented in Chapter 4 of this dissertation.

Experimental reproduction of MHR-associated disease has varied greatly. Intra-nasal (IN) and intraperitoneal (IP) inoculations have been the administration methods of choice in past experiments, with IP showing the most consistent results (18, 27, 29, 30, 32, 33, 37, 39). Intra-tracheal (IT) administration has also been reported (36). With the exception of Chen *et al.* (36), the majority of the MHR challenge models were developed nearly 20 years ago, with some studies conducted in the early 1970's. Some of these studies were conducted with very low numbers of animals (n=5, in some cases). Additionally, clinical signs could vary greatly between challenged animals with some animals showing polyserositis and no lameness, some showing both polyserositis and lameness, and some unaffected. Herd genetics, vaccine and antibiotic protocols, and mycoplasma strain variations have undoubtedly changed since the earliest MHR investigations and each may play a role in disease outcomes. An MHR

challenge model which consistently and effectively produces both polyserositis and lameness in a number of animals sufficient to provide power for statistical analyses in future vaccine trials is lacking. Chapter 3 of this dissertation addresses this need.

Phase shifting and antigenic variation

Due to their metabolic requirements necessitating a parasitic lifestyle, MHR membrane components are vital for interactions with host cells and nutrient acquisition. Whereas Gram-positive bacteria have a thick peptidoglycan layer in their cell wall and Gram-negative bacteria have an inner and outer membrane separated by a periplasmic space, the lack of a cell wall in *Mycoplasma* leaves them more vulnerable to attack by the host immune cells. *Mycoplasma hyorhinis* possess a unique set of membrane-associated variable lipoproteins (Vlp) which can allow them to evade immune attack (1, 40-44).

To date, seven Vlp genes have been identified (*vlpA – vlpG*) with individual strains of MHR possessing different repertoires of these genes (45-49). Of the six MHR genomes sequenced to date, the tissue culture derivatives and the broth derived clonal variant are lacking one or more of the seven known *vlp* genes.

The Vlp system for MHR has been closely examined. Rosengarten and Wise (41) demonstrated heterogeneity in phenotypes of an *in vitro* cultured, clonal MHR isolate, with colony morphology varying in size and opacity. These colonies also demonstrated the phenomenon of sectoring for both morphologies. Immunoblotting revealed phase switching in lipoprotein expression (41). Further research has shown a diverse population of Vlp's with phenotypes varying in both their size and their expression (41, 43). The source of variability has been attributed to high-frequency, random mutations in these chromosomally clustered

vlp genes. Indel mutations in each of the *vlp* promoters lead to variability in the transcription of each gene and thus, phase variation in Vlp expression (40, 43, 50, 51). Similarly, random mutations can affect the size of each Vlp via intragenic recombination in tandem sequence repeats present in 3' regions of the *vlp* genes (40, 43). Citti *et al.* (43) established that the longer Vlp size variants were able to negate complement-independent antibody inhibition *in vitro* and these variants were selected for when cultured in the presence of these antibodies (43). Thus, at least some of the Vlp's are capable of providing protection for MHR against humoral attack. The lack of this selective pressure may be a reason for their absence in the sequenced *in vitro* (cell- and broth-derived) isolates (46-48).

The variable lipoproteins represent a heritable system in MHR leading to a diverse population and capable of providing protection for the mycoplasma.

Pathogenesis and virulence factors

As with the other swine mycoplasmas, pathogenesis for MHR is not fully understood. It is thought the pathogen enters the host via the respiratory route as MHR colonizes the upper respiratory tract. Few specific colonizing factors have been identified. Proteins involved in attachment to ciliated epithelial cells have been described for MHP (52) and include the well-studied protein P97. There is a functional ortholog of this protein, P95, in MHR which may play a similar role for this species (53).

As described above, the Vlp system has been implicated in immunoevasion and immunosuppression. Presentation of highly variable surface antigens to the host requires a similar, constantly changing supply of antibodies to combat. As Citti *et al.* demonstrated (43), these lipoproteins appear to also be decoy antigens, preventing antibody inhibition.

Adsorption to mammalian cells *in vitro* has been well documented for several species of *Mycoplasma* (54, 55). The process of adsorption for MHR has been shown to involve cap formation (56) and can be independent of sialic acid binding (57). A trypsin sensitive protein of approximately 71 kDa has been linked with MHR adhesion to mammalian cells (58). Adhesins for the human pathogen *Mycoplasma pneumoniae* have shown sequence homology to mammalian structural proteins (59). It is this molecular mimicry which may be involved with autoimmune-like disease manifestations and overwhelming inflammatory responses in the host following infection with *M. pneumonia* (59). These responses are similarly observed in systemic MHR infections and may indicate such mimicry exists in this species. The exchange of membrane components has been observed between adherent MHR and experimentally infected cells which may lead to modifications in the host cells' functions for metabolism and interactions with the extracellular matrix (60, 61).

Intracellular growth has been established in several *in vitro* cell lines (48, 57, 62, 63). Receptor-mediated endocytosis has been suggested as one means of entry following adhesion to the cell surface (63, 64). Interactions between the high-affinity MHR transport protein p37 and host cell Annexin A2 have been implicated for mammalian cell invasion (65). Yuan *et al.* (2016) inhibited MRH infection of gastric cells by interrupting this interaction via competitive binding (66). Despite these studies, the exact mechanisms required for cellular invasion remain vague.

There are conflicting reports as to MHR survival upon cellular invasion. Hu *et al.* suggests an endosomal niche by disruption of late endosomal formation and inhibition of autophagocytosis (63). Yet Chernov *et al.* suggest that MHR avoids the endosomal pathway altogether and persist within the exocyst, also providing a means for escape (67). This later

group further demonstrated variations in methylation of CG and GATC sites between internalized and external MHR and proposed preferential selection of methylation patterns by MHR based on environment (67).

Some mechanisms for nutrient acquisition have been identified. With no ability for *de novo* nucleic acid synthesis, MHR uses enzymes such as cytidine deaminase, pyrimidine nucleoside phosphorylase, purine nucleoside phosphorylase, and thymidine phosphorylase (68-70) to catabolize the building blocks for DNA synthesis. Membrane associated nucleases (71) and oligonucleotide transporters (64) have been identified which aid in the acquisition and transport of required molecules. None of the mycoplasmas can synthesize or modify long-chain fatty acids and must acquire them from the environment. *Mycoplasma hyorhinis* shows a slight affinity for palmitic acid over oleic acid when these fatty acids are radiolabeled and measured after incorporation into membrane lipids (11). There are enzymes in the MHR repertoire for *de novo* phospholipid generation and include triacylglycerol lipase, glycerol-3-phosphate acyltransferase, and cardiolipin synthetase. The fatty acid transporters have not been fully identified. However, such proteins have been associated with virulence in *M. pnemoniae* (72).

Immune response

With pathogenesis poorly defined, researchers have looked to immunological responses in the host to aid in defining the infection process for MHR.

Serum-based ELISA's are generally the most economical of diagnostic assays for both the producer and testing labs due to the relative ease of sample collection and well established assay principles. However, serological assays for MHR and MHS have not been

widely available. There are no less than four commercial ELISA's for MHP. Although there has been a poor correlation to results from these assays and the severity of MHP disease, they provide a good indication of herd status (73-75). Neto *et al.* demonstrated that cross-reactivity among MHR, MHP, MHS, and MF antibodies was possible in similarly prepared assays (73). These facts have made the development of a reliable ELISA-based assay for the detection of MHR antibodies difficult.

Ross *et al.* demonstrated that complement-fixing antibodies to MHR could be detected in the serum of experimentally infected pigs as early as two weeks post-inoculation and up to one year after, with the majority of these antibodies being IgG (34). They further showed these same antibodies could also be detected in the synovial fluid from arthritic joints of the infected pigs and, generally, in higher quantities than those found in serum which is in accordance with the results of Barden and Decker (34, 76). Recently, MHR IgA antibodies have also been shown to be detectable in pen-based oral fluids (73). The establishment of a humoral response to MHR infection in swine is clear. The passive transfer and length of maternal immunity have not yet been studied. The prolonged detection of these MHR antibodies may likely indicate a chronic infection in the animal.

Acute septic arthritis by *Staphylococcus aureus* may provide insight into the chronic arthritis noted with MHR. There is no basement membrane underlying the synovial membrane which is well-vascularized and provides a point for bacterial entry (77). Extracellular matrix host proteins such as collagen and fibronectin facilitate adhesion (77). Following adhesion, *S. aureus* can be internalized by the host cell by methods such as receptor-mediated endocytosis. Once inside the cell, the bacteria can either induce apoptosis or persist within the host cell. Apoptosis may lead to the damage observed with septic

arthritis while persistence in the cell can allow for immunoevassion of inflammatory cells (77). The local inflammatory response in the joint begins with a cytokine response including the release of IL-1β and IL-6 into the synovial fluid, activating the acute-phase liver proteins which bind the bacteria for opsonization and complement activation. The inflammatory response continues with the release of TNFα and IL-8 as well as the reactive oxygen species nitric oxide (77).

Access to the synovial membranes by MHR is likely similar to *S. aureus* with a hematogenous entry. *Mycoplasma hyopneumoniae* has defined heparin, fibronectin and plasminogen binding factors (78, 79) and such similar mechanisms may be likely found in MHR as putative adhesions. Invasion into the host cell via receptor mediated endocytosis (63-65) and intra-cellular survival are discussed above. Similar to the *S. aureus* response, in pigs bred for a high immune response and experimentally inoculated with MHR, an increase in the expression of IL-8, IL-10 and TNFα were observed in mononuclear cells of the synovial membranes (80). Human monocytes have also been shown to secrete TNFα when experimentally infected with MHR *in vitro* (81).

Obara *et al.* has provided evidence that human gastric cells infected *in vitro* with MHR undergo drastic physical changes which can be attributed, in part, to the bacteria's endonucleases, as well as increased cellular nitric oxide (NO) and up-regulation of the inducible nitric oxide synthase (iNOS) (82). The final outcome of this increase in NO is anoikis, defined as apoptosis due to a cell's detachment from the extracellular matrix and inability to persist without cell-to-cell signaling. Anoikis increased as NO levels increased, and detachment from the matrix was shown to be due to NO attenuation of E-cadherin, an epithelial adhesion molecule (82). The effect of anoikis in the joint of an infected pig may

lead to an accumulation of dead epithelial cells in the affected area. Removal of this material would require an inflammatory response and can be difficult to accomplish in cartilaginous tissue, prolonging the inflammation and leading to arthritic-like symptoms (83).

Nitric oxide (NO) promotes interferon gamma (IFN-γ) in T helper 1 (Th1) cells and drives T-cell differentiation towards the Th1 phenotype (84). The result is an imbalance in Th1 and Th2 cells, a hallmark of chronic inflammatory diseases such as rheumatoid arthritis (84). This T-cell disparity further results in lower B cell activation and may provide one explanation for the discrepancies observed between serological assays and disease severity as described for MHP.

Toll-like receptors (TLR) play a crucial role for innate immunity. The two TLR's associated with lipoproteins, TLR2 and TLR6, have been shown to play a role in MHP-associated disease (85). *In vitro* studies have shown a pro-inflammatory response to MHR infection to be at least partially associated with TLR2 (86); however, TLR4, which is normally associated with and binds to lipopolysaccharide (LPS), has also been shown to activate inflammatory genes (87, 88). This TLR4 association has been demonstrated to be mediated through the MHR protein p37 (87, 88).

There has been some debate as to whether enhancement to cell-mediated or humoral immunity is more beneficial in response to mycoplasma infection. Investigations with the mouse model for *M. pulmonis* led Cartner *et al.* to hypothesize that innate immunity is preferred for protection of the lungs and respiratory mycoplasmas but humoral immunity provided for the best protection against systemic mycoplasmal disease (89). By this measure, a strong antibody response in pigs would help alleviate systemic MHR infection.

Tissue culture

Mycoplasma hyorhinis has been a well-known contaminant of mammalian tissue cultures for over 50 years (48, 56, 57, 62, 86, 90-94). This fact has been utilized to cultivate previously “non-cultivatable” strains of MHR (91). The growth factors involved in such cultivation techniques have not yet been definitively identified. Tissue culture has been used to demonstrate the invasive ability of some *Mycoplasma* species, including MHR, which were previously thought to remain on the surface of mammalian cells (48, 57, 62, 64). There have been many proposed sources for covert MHR tissue culture contamination. These include animal derived culture reagents (e.g. porcine trypsin), media supplementation (e.g. animal serum), or the humans that work with such cell lines (90, 95).

The insidious nature of undetected mycoplasma contamination has led to a commercial niche devoted entirely to detection and eradication of such contamination. The United States Department of Agriculture (USDA) requires testing for the detection of mycoplasma contamination in vaccine components per title 9, *Code of Federal Regulations* (CFR), section 113.28 to ensure purity of vaccines. Such rigorous screening is not necessarily employed in all laboratories. Contamination by mycoplasmas such as MHR has been shown to alter normal growth traits in primary and immortal cell lines (86, 90, 95). Investigators must keep this in mind and ensure tissue culture-based experiments are not misinterpreted due to undetected mycoplasma contamination.

Cancer

Recent research has focused on the putative link between MHR and human cancers. Antibodies to MHR, specifically targeted to the p37 protein, have been found in prostate cancer patients (96). It has not been definitively demonstrated whether MHR infection in these patients has resulted in the cancer or, conversely, if it is the cancer that has suppressed these individuals enough to allow for MHR infection to occur. As described above, MHR can easily infect mammalian cells *in vitro*; however, isolation from human *in vivo* samples has not been established. It should be said that MHR is considered a pathogen of swine and therefore a medical practitioner would have little reason, until recently, to attempt recovery of this bacteria in diagnostic samples. *In vitro* studies using cancer cells have shown that MHR can increase cell migration and invasion and has been positively correlated to tumor metastasis in gastric cancer tissues (88, 97, 98). These studies have focused in particular on the MHR p37 protein.

A member of a high affinity transport system, p37 has been structurally examined and found to bind thiamine pyrophosphate (TPP) (99). It is not known what role this sequestration of thiamine plays in cell culture systems or in the host. Inoculation of cell cultures with p37 have been shown to activate NF-κB, a transcription factor for several inflammatory and cancer-associated genes (65, 87, 88, 100). The exact mechanism that allows for increase invasivity of cancer cells is unknown.

Besides the implications with oncogenesis, MHR has also been shown to interfere with cancer therapies. Purine and pyrimidine nucleoside analogues have been used for treatment of cancers (68, 70, 101, 102). *Mycoplasma hyorhinis* possess enzymes which can

catabolize these nucleoside analogues, thus, inactivating these drugs and adversely affecting their efficacy (68-70, 102).

The possibility of human MHR infection and the ensuing implications with cancer necessitates caution when handling the organism in the laboratory and potentially infectious tissue in production and diagnostic settings.

Summary with Hypothesis and Goals

The lack of a commercially available vaccine to aid in MHR disease management has relegated producers to a heavy regime of antibiotics to alleviate disease parameters. The overall goal of this research is to provide a viable MHR vaccine candidate. As such, a robust, consistent and reproducible disease model was needed to fully evaluate these candidates in a manner acceptable to regulatory authorities.

The central hypothesis is that MHR infected tissue culture resembles *in vivo* infection and, therefore, protein expression would more closely mimic that expressed in the pig and would provide for both virulent challenge material and efficacious vaccine antigen.

The experiments discussed in Chapter 2 detail challenging caesarian-derived, colostrum-deprived (CDCD) pigs with cell-associated MHR. The observation of lameness during the challenge period and the presence of pericarditis at necropsy were noted. The dose and route of administration of this material were optimized. The experiment described in Chapter 3 then examined the susceptibility of CDCD animals to this challenge model at incremental increases in age, from seven weeks through sixteen weeks of age.

In Chapter 4, three doses of an inactivated, whole-cell vaccine were administered to pigs and compared to a product matched, mycoplasma-free placebo for efficacy in the previously established challenge model. Pigs were evaluated for lameness and pericarditis.

Chapter 5 is a proteomic analysis comparing two *ex vivo* cultures of MHR to a broth culture for differential protein expression. Madin-Darby canine kidney cells (MDCK) and McCoy mouse fibroblast cells were infected with a single isolate of MHR. These cultures were then examined by liquid chromatography, tandem mass spectrometry (LC-MS/MS) along with a standard broth culture of the same MHR isolate.

The final chapter is a general conclusion and includes discussions for future work. The tissue culture-associated, inactivated whole-cell vaccine provided significant reductions of both lameness and pericarditis. This vaccine will help alleviate disease in the production setting. It will also provide a benchmark for future vaccine trials, evaluating target candidates identified by the proteomic analysis.

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CHAPTER 2. DEVELOPMENT AND OPTIMIZATION OF A CELL-ASSOCIATED CHALLENGE MODEL FOR *MYCOPLASMA HYORHINIS* IN SEVEN-WEEK OLD CAESAREAN DERIVED-COLOSTRUM DEPRIVED PIGS

A paper prepared for submission to a peer reviewed journal

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Abstract

Mycoplasma hyorhinis (MHR) causes polyserositis and lameness in grower pigs. While herd-specific vaccines are being marketed, no fully licensed vaccine is available in the regional or global market. In order to evaluate suitable vaccine candidates, we have developed a challenge model in caesarean-derived colostrum-deprived pigs using cell-associated MHR which results in both severe pericarditis and lameness. We investigated the administration of MHR to seven-week-old pigs over three days using three different routes compared to a single day administration using three different routes. Pigs were monitored for twenty-one days for signs of lameness and well-being. At study termination, pigs were examined for evidence of *Mycoplasma*-associated polyserositis and arthritis. Results indicate that clinical manifestation of disease was more dependent on the route of administration than the total given dose. A single intravenous (IV) administration resulted in extensive polyserositis while a single intranasal (IN) administration showed little-to-no signs of disease. A single intraperitoneal (IP) administration did not induce the same level of

polyserositis as observed in the IV group, but did result in an increased incidence of lameness compared to this group. Further, pigs administered MHR by IP (Day 0), IV (Day 1), and IN (Day 2) routes on three consecutive days showed a more robust disease manifestation, resulting in both polyserositis and lameness. Optimization of this group showed that elimination of the third-day IN challenge had no detrimental effect on clinical outcomes. The consecutive-day administration of cell-associated MHR will allow for simultaneous evaluations of both polyserositis and lameness in future vaccine trials.

Introduction

Mycoplasma hyorhinis (MHR) is commonly found in the upper respiratory tract of pigs and can cause respiratory disease, polyserositis, and polyarthritis in animals typically around seven weeks of age (1, 2). The mechanisms of pathogenesis of this ubiquitous organism, including translocation from colonized upper airway to systemic dissemination in the animal, and the mode of targeting of specific organs, are currently unknown.

Reduced productivity due to MHR-associated disease has economic consequences in the swine production setting (3). Prophylactic and therapeutic antibiotic treatment can mitigate *Mycoplasma* infections (2, 3), but there is a desire in the swine industry to move away from such treatments (4, 5). There are currently no licensed, commercially available vaccines for MHR. In order to develop and evaluate potential vaccine candidates, a robust and effective challenge model needs to be established. As the pathogenesis of MHR is poorly understood, such a model would also help to increase our understanding of the infection process and development of disease. Previous experimental inoculation protocols for swine Mycoplasmas using standard, broth-derived material have resulted in varying degrees of

polyserositis and lameness (6, 7). However, the low number of animals tested and the inconsistency of affected animals necessitated further investigation. To this end, we have developed a suitable model, which consistently results in pericarditis and lameness in caesarean-derived colostrum-deprived (CDCD) pigs following infection with tissue-culture derived MHR.

Materials and Methods

Two studies are discussed herein. The objective of Study 1 was to evaluate three different single routes of administration and two multiple route, consecutive-day administration of MHR for production of polyserositis and lameness. The objective of Study 2 was to compare the multiple route, consecutive-day administration with and without the third-day, IN challenge for production of polyserositis and lameness.

Challenge material

The MHR challenge isolate originated from the joint fluid of a clinically affected pig. After initial isolation and speciation, the bacteria were grown in modified Friis media (8) using established methods. To generate the infection dose of MHR, confluent monolayers of MDCK (Madin-Darby canine kidney) cells were infected as follows. Media for cell maintenance was minimum essential media (MEM; Gibco/Thermo Fisher Scientific Inc., Waltham, MA) supplemented with 5% fetal bovine serum (FBS; SAFC, St. Louis, MO). Prior to infection, media was removed, and cells were rinsed with 0.1 M phosphate buffered saline (PBS; Thermo Fisher Scientific Inc., Waltham, MA). *Mycoplasma hyorhinis* were then added to the tissue culture at approximately 10% (v/v) of the total final volume and allowed to incubate at 37°C for at least two hours prior to adding pre-warmed media consisting of

MEM supplemented with 2% FBS. Harvest of the infected cell layer was performed after observation of cytopathic effect (CPE), generally occurring around four to seven days post-infection, by freezing the infected cells at ≤ -60°C, thawing at 37°C, and then collecting the lysed cell-suspension. MHR challenge stocks were combined with sterile glycerol (10%, v/v) prior to dispensing into working volumes and stored at ≤ -60°C until challenge. Challenge doses of MHR were quantified by color changing units (CCU).

Animal information

CDCD piglets were purchased from a commercial Class A dealer and were a commercial cross breed. Both gilts and intact barrows were used. Animals were determined to be free from MHR and *M. hyosynoviae* (MHS) colonization as determined by real-time PCR analysis of nasal swabs (eSwab™, Copan; Murrieta, CA) collected prior to challenge. Nasal swabs from Study 2 animals were also negative for *M. hyopneumoniae* (MHP) by real-time PCR; Study 1 animals were not tested. Pre-challenge sera were determined to be free from MHP antibodies by ELISA (IDEXX, Westbrook, ME). All animals were seven weeks ± 5 days of age at the time of initial inoculation. All animals were deemed to be in good health and nutritional status before the studies were initiated. No biologicals or pharmaceuticals other than the challenge material were administered to the test animals after arrival at the study site. Non-medicated feed was used throughout the duration of each study. Feed rations were appropriate for the age, condition, and species of test animal. Water was provided *ad libitum* throughout each study. Both studies were performed in BSL-2, USDA inspected facilities following the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (9).

For Study 1, pigs were blocked by litter and randomly assigned to five groups of ten animals each for experimental challenge, labeled groups 1-5. One group of six pigs was used for non-infected, negative controls and labeled group 6. Animals were housed in three rooms in pens on raised decks with metal slated floors. To reduce the chance of nasal shedding, those groups receiving IN challenge at any time (group 3-5) were house in one room and grouped by pen. Those groups not receiving IN challenge (group 1 and 2) were housed in another room and grouped by pen. The negative controls (group 6) were housed in a separate room in a single pen.

For Study 2, pigs were blocked by litter and randomly divided into two groups of 28 animals each for experimental challenge, labeled group 1 and 2. A third group of eight pigs was used for non-infected, negative controls. Animals were housed in two rooms in pens on raised decks with metal slated floors. Challenged animals (group 1 and 2) were housed in one room, co-mingled in four pens and the negative control animals (group 3) in a separate room in one pen.

Experimental inoculation

The inoculation procedure for Study 1 was as follows. At study day 0 (D0), pigs in group 1 received 40 mL of MHR by intraperitoneal (IP) route at a dose of 1.59×10^9 CCU/pig. Pigs in group 2 received 20 mL of MHR by intravenous (IV) route at a dose of 7.96×10^8 CCU/pig. Pigs in group 3 received 20 mL of MHR by intranasal (IN) route (10 mL/nostril) at a dose of 7.96×10^8 CCU/pig. Pigs in group 4 received MHR at 40 mL/IP (1.59×10^9 CCU/pig) on Day 0, 20 mL/IV (8.53×10^8 CCU/pig) on Day 1, and 20 mL/IN (4.18×10^8 CCU/pig) on Day 2, for a total dose of 2.86×10^9 CCU/pig. Pigs in group 5

received MHR at 20 mL/IP (7.96×10^8 CCU/pig) on Day 0, 10 mL/IV (4.27×10^8 CCU/pig) on Day 1, and 10 mL/IN (2.09×10^8 CCU/pig) on Day 2, for a total dose of 1.43×10^9 CCU/pig; half the dose of group 4. Animals in group 6 received no treatment. The challenge scheme for Study 1 can be seen in Table S1 in the Appendix A.

The inoculation procedure for Study 2 is as follows. Pigs in group 1 received MHR at 20 mL/IP (1.12×10^9 CCU/pig) on Day 0, 10 mL/IV (6.76×10^8 CCU/pig) on Day 1, and 10 mL/IN (1.00×10^8 CCU/pig) on Day 2, for a total dose of 1.90×10^9 CCU/pig. Pigs in group 2 received MHR at 20 mL/IP (1.12×10^9 CCU/pig) on Day 0 and 10 mL/IV (6.76×10^8 CCU/pig) on Day 1, for a total dose of 1.80×10^9 CCU/pig. The challenge scheme for Study 2 can be seen in Table S2 in the Appendix A.

Clinical observations

Pigs were observed daily from the first day of challenge through study termination for clinical signs of disease, respiratory distress, and lameness. Respirations and coughing were scored as 'normal' (=0) or 'abnormal' (=1). Lameness was scored as 'normal' (=0), 'mild' (=1), 'moderate' (=2), 'moderately severe' (=3), 'severe' (=4), or 'recumbent" (=5). Definitions for the lameness categories were as follows: **normal**—no visible lameness; **mild**—not constantly lame when walking, walking at a normal speed, weight bearing while walking and standing, lameness indicated by intermittent reduced weight bearing on one limb or shortening of the stride; **moderate**— lameness constant and observed throughout every step at a walking pace, bearing some weight on the leg at a walk and standing but short-striding one or more legs while walking, walking at a normal speed; **moderately severe**—non-weight bearing on the leg the first few steps after standing, constant, obvious lameness

while at a walking pace, putting very little weight on the leg at a walk or while standing, requiring the pig to slow its speed of walking; **severe**— will stand (may require assistance) for at least 3 minutes, non-weight bearing on one or more legs at walk or standing, still able to three-legged walk; and **recumbent**— will not stand even with assistance. A pig was considered lame if it received a score ≥ 1 on two or more consecutive observation days. The clinical observation scoring systems can be seen in Table S3 in the Appendix A.

Weight measurements were collected just prior to challenge and just prior to euthanasia. At study termination, all pigs were anesthetized and then euthanized by electrocution.

The experimental schedules of events are summarized in Table S4 and S5 in the Appendix A for Study 1 and 2, respectively.

Gross pathological examination and sample collection

After euthanasia, a necropsy and gross pathological examination were performed for all animals. The thoracic and abdominal cavities were examined for evidence of polyserositis (pleuritis, pericarditis, and peritonitis). In Study 1, heart tissue was collected from all pigs and tested for MHR and MHS by real-time PCR. Affected lung tissue was also collected. If no affected lung tissue was present, a section of the right diaphragmatic lobe was collected. Lung tissue was tested for MHR and MHS by real-time PCR. In Study 2, in lieu of tissue collection, pericardial swabs (eSwab™, Copan; Murrieta, CA) were taken from all pigs, and pleural swabs were taken from those animals exhibiting pleuritis. All swabs were tested for MHR, MHS and MHP by real-time PCR in both studies. The elbows, stifles, carpi and tarsi were opened and examined for signs of arthritis (e.g. excess synovial fluid, abnormal

synovial fluid, abnormal synovial membranes, abnormal articular surfaces). For each individual pig, a single, common swab (eSwab™, Copan; Murrieta, CA) was used to sample the articular surfaces of both elbows and both stifles. A second swab was used to sample the articular surfaces of all four carpi and tarsi. All joint swabs were tested for MHR, MHS, and MHP by real-time PCR.

Histopathology

For Study 1 only, lung and heart tissues were collected from all pigs and fixed in 10% neutral buffered formalin. Samples were submitted to Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL) for histological examination (haematoxylin and eosin staining). Pleuritis was scored as none (=0), mild (=1), moderate (=2), or severe (=3) as were peribronchiolar/septal infiltrates. Heart tissue was scored for lymphocytic inflammation (0 = no inflammation, 1 = mild accumulation of lymphocytes, 2 = moderate accumulation of lymphocytes, 3 = severe accumulation of lymphocytes) and fibrosis of the epicardium (0 = no fibrosis, 1 = mild, 2 = moderate, 3 = severe).

Real-time PCR

Total nucleic acid extractions were performed with a Qiagen BS96 Vet 100 BioSprint using the One-For-All Vet Kit (384) (Qiagen, Venlo, Limburg). Mycoplasma species-specific regions of the 16S rRNA gene were then detected by real time PCR. The PCR mix contained 2x SsoAdvanced™ probe supermix (BioRad, Hercules, CA), 80 µM of each primer, 50 µM of probe, and 2 µL of DNA in a total volume of 20 µL. See Table 2.1 for primers, probes and control oligonucleotides sequence information. DNA amplification was carried out using a BioRad CFX96 Real Time System (BioRad, Hercules, CA) with the

following conditions: 2 min at 95°C then 35 cycles of 5 sec at 95°C and 5 sec at 58°C. Pure cultures of MHR and MHS were used as positive controls. For quantification, Ct values of the samples were compared to the standard curve. The standard curve was elaborated with serial dilutions of a synthesized control oligonucleotide of known DNA concentration. A Ct value of < 35 was considered positive. The detection limit of these assays is ~1000 genomic copies/mL of sample. The method for MHP detection by real-time PCR has been previously described (10).

Table 2.1. List of Primers, Probes, and Control Oligo Sequences

PCR	Name	Sequence
M. hyorhinis	MycoR-probe	5'-6-FAM-AGCTGTGAAGCTCCTTTCTATTACTCATC-BHQ_1/-3'
	MHR-F	5'-GCTAATACCGGATATAGTTATTT-3'
	MHR-R	5'-GCACCCCCATTTTTAAG-3'
	Control	5'-TTGGAAACAATAGCTAATACCGGATATAGTTATTTATCGCATGATGAGTAATAGAAAGGAGCTTCACAGCTTCACTTAAAAATGGGGGTGCGGAACATTAGTTA-3'
M. hyosynoviae	MycoS-probe	5'-Cy5-AAGCAAACGCTTCTTTCATAACGAAATC-BHQ_2-3'
	MHS-F	5'-TTAATGCCGGATAAGTATGAA-3'
	MHS-R	5'-GCACCCTCATCTCTTAG-3'
	Control	5'-GGAAACATTGGTTAATGCCGGATAAGTATGAAATCGCATGATTTCGTTATGAAAGAAGCGTTTGCTTCACTAAGAGATGAGGGTGCGGAACATTAGC-3'

Bacteriology

To exclude contribution by extraneous bacterial pathogens in the study animals, all samples collected at the time of necropsy were cultured onto sheep blood agar (Remel/Thermo Fisher Scientific Inc., Waltham, MA) with a *Staphylococcus* "nurse" streak for aerobic and anaerobic growth. Plates were incubated at 37° C for two days and examined daily for growth. Further isolation, if necessary, was performed on colonies of interest and identification was performed by ISU-VDL.

Statistical analysis

Statistical summaries were conducted by a BIVI statistician using SAS Version 9.4. Summary tables can be found in Appendix A.

For Study 1, treatment was confounded by housing and results are therefore summarized by treatment group. Individual average daily gain during the 21-day period was calculated by subtracting day 0 weight from day 21 weight and dividing by 21. Day 0 weight, day 21 weight, and average daily gain for each treatment group was summarized using the MEANS procedure of SAS. Means, standard errors, and 95% confidence intervals for each treatment were determined (Table S6). Polyserositis, any joint showing signs of arthritis, and PCR results were each scored as positive or negative. These variables were each summarized by treatment group using the FREQ procedure of SAS. The proportion positive, standard error and Clopper-Pearson Exact 95% confidence interval were determined for each treatment group (Tables S7, S8, and S9). Distributions of lameness scores were summarized by day and treatment group using the TABULATE procedure of SAS. A lameness score ≥ 1 for any two consecutive days during the study was analyzed as a binomial outcome (positive

if true, negative if false). Results were summarized by treatment group using the FREQ procedure of SAS. The proportion positive, standard error and Clopper-Pearson Exact 95% confidence interval were determined for each treatment group (Table S10). Distributions of histological scores were summarized by treatment group using the TABULATE procedure of SAS. In addition, binomial outcomes were created for each variable (positive if score > 0, negative if score=0). These binomial outcomes were each summarized by treatment group using the FREQ procedure of SAS. The proportion positive, standard error and Clopper-Pearson Exact 95% confidence interval were determined for each treatment group (Table S11).

For Study 2, treatment groups 1 and 2 were housed together and therefore, statistical comparisons include group 1 versus 2. Individual average daily gain during the 28-day period was calculated by subtracting day 0 weight from day 28 weight and dividing by 28. The analysis utilized the MIXED procedure of SAS. The model included the fixed effect of group and the random effects of litter and pen within group. Least squares means, standard errors, and 95% confidence intervals for each treatment were determined as well as comparisons of group 1 versus group 2 (Table S12). Polyserositis evaluations were summarized using the TABULATE procedure. Evaluations of peritonitis were all negative. Pericarditis and pleuritis were analyzed by room using the GLIMMIX procedure with binomial distribution and logit link. The model included the fixed effect of group and the random effects of litter and pen within group. Back transformed least squares means, standard errors, and 95% confidence intervals were determined as well as comparisons of group 1 versus 2 (Table S13). PCR swabs were analyzed as binomial outcomes. Pericardial, pleural, elbow/stifle, and carpal/tarsal swabs were each analyzed using the GLIMMIX procedure with binomial

distribution and logit link. The model included the fixed effect of group and random effects of litter and pen within litter. Back transformed least squares means, standard errors, and 95% confidence intervals were determined as well as comparisons for group 1 versus 2 (Table S14). Presence of arthritis for each location (left and right elbow, left and right stifle, and left and right tarsal) were analyzed using the GLIMMIX procedure with binomial distribution and logit link. The left and right carpal was free from arthritis in all animals. The model included the fixed effect of group and random effects of litter and pen within litter. Back transformed least squares means, standard errors, and 95% confidence intervals were determined as well as comparisons for group 1 versus 2 (Table S15). In addition, the presence of arthritis at any location was also analyzed using a similar model (Table S15). For respiratory and coughing observations, the presence or absence of the sign any time during the 28-day period was determined and analyzed. The GLIMMIX procedure with binomial distribution and logit link was utilized for analysis. The model included the fixed effect of group and random effects of litter and pen within litter. Back transformed least squares means, standard errors, and 95% confidence intervals were determined as well as comparisons for group 1 versus 2 (Table S16). In addition, lameness scores ≥ 1 for any two consecutive days was also analyzed using the same approach (Table S16).

Results

Clinical observations

In Study 1, no abnormal respirations or coughing were noted in any pig in any group. Lameness was noted in animals as early as five days post-challenge. One animal (10%) in each group 2, 3, and 4 was scored as lame. Of the single lame animals in each group 2 and 3, both animals received a score of "1" on two consecutive days: Days 20-21 for the pig in group 2 and Days 18-19 for the pig in group 3. The single lame animal in group 4 received scores of "2", "3", "1" and "1" on Days 8, 9, 17, and 18, respectively. Group 1 and group 5 had the highest incidences of lameness with 40% and 70% affected, respectively. The four lame animals in group 1 were scored as lame on multiple days, with scores ranging from "1" to "3": one animal receiving a score of "1" or "2" on Days 14-21; one with scores of "1 or "2" on Days 6-7 and Days 12-14; one with scores of "1" or "2" on Days 15-21; and the last with scores of "1" to "3" on Days 16-21. The seven lame animals in group 5 all received scores on multiple days, with scores ranging from "1" to "4": one animal receiving a score of "1" or "2" on Days 12-21; one with scores of "1" on each of Days 17-21; one with a score of "3" on Day 9 and then scores of "1" on each of Days 10-13 and Days 15-19; one with scores of "1" or "2" on Days 16-21; one with a score of "1" on each of Days 19-21; one with a score of "4" on Day 7, "2" on Day 8, and "1" on each of Days 15-21; and one with a score of "1" on each Days 15-16 and a score of "2" on Day 20. All non-infected group 6 animals remained clinically normal throughout the duration of the study. See Figure 2.1A for a summary of the percentage of animals positive in the challenged groups.

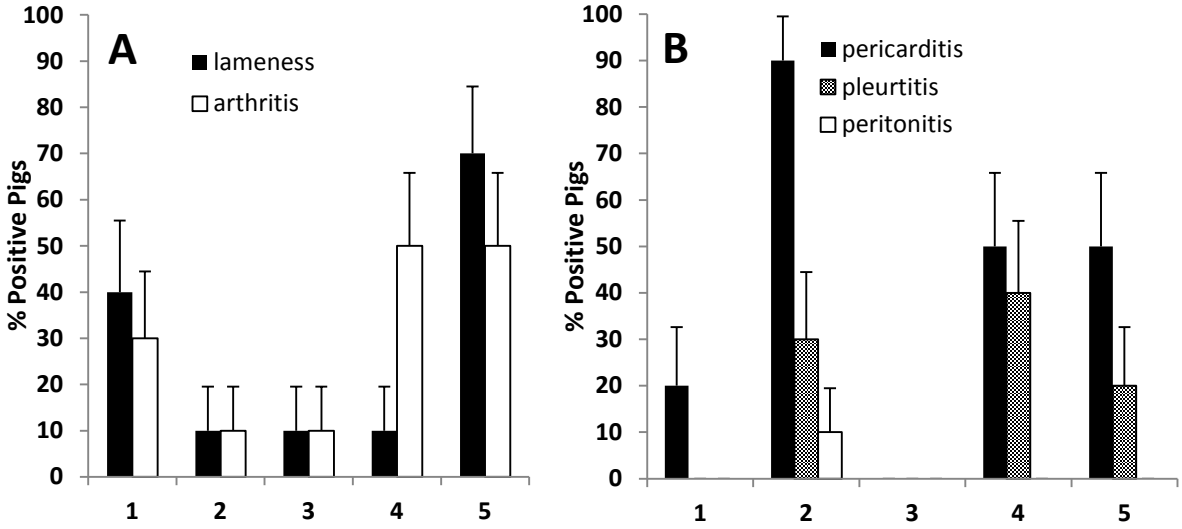

Fig. 2.1. Study 1 Clinical evaluations by group. A. Assessment for lameness and arthritis. **B**. Assessment for polyserositis. Groups: 1=IP (intraperitoneal), 2=IV (intravenous), 3=IN (intranasal), 4=IP, IV, IN (high dose), 5=IP, IV, IN (low dose). All group 6 non-infected controls remained free from clinical findings and are not represented. Data represents the percent (%) positive animals ± standard error.

The average daily gain (ADG) for Study 1 was calculated for all groups as shown in Figure 2.2. With the exception of group 3, all challenged groups had a similar average daily gain with group 5 showing the lowest average of 0.4 kg/day. Group 1, 2 and 4 each had an average of 0.5 kg/day. Group 3 and the non-infected group 6 each had an average of 0.7 kg/day.

Figure 2.3A shows the distribution of clinical observations from Study 2. Abnormal respirations were noted in 64.3% of the pigs in group 1 and 71.4% of the pigs in group 2. Animals began showing signs of abnormal respirations on Day 10 and Day 11 for group 1 and 2, respectively, and continued intermittently for both groups through study termination. Coughing was minimal with 14.3% of the pigs in group 1 and 10.7% of the pigs in group 2 affected. Coughing scores were noted in both groups on four sporadic days each. Lameness was observed in 82.1% of the pigs in group 1 and 75.0% of the pigs in group 2 and was noted

in both groups as early as the second study day and continued through study termination. Lameness scores ranged from “1” to “3” throughout the observation phase. All non-infected group 3 animals remained clinically normal throughout the duration of the study.

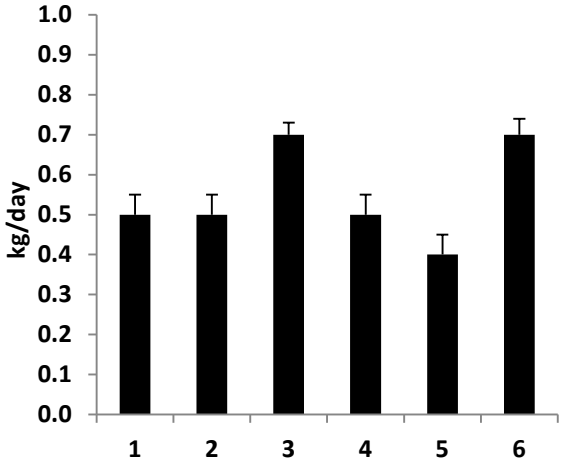

Fig. 2.2. Study 1 average daily weight gain by group. Groups: 1=IP (intraperitoneal), 2=IV (intravenous), 3=IN (intranasal), 4=IP, IV, IN (high dose), 5=IP, IV, IN (low dose), 6=no treatment. Data represents the mean kg/day ± standard error.

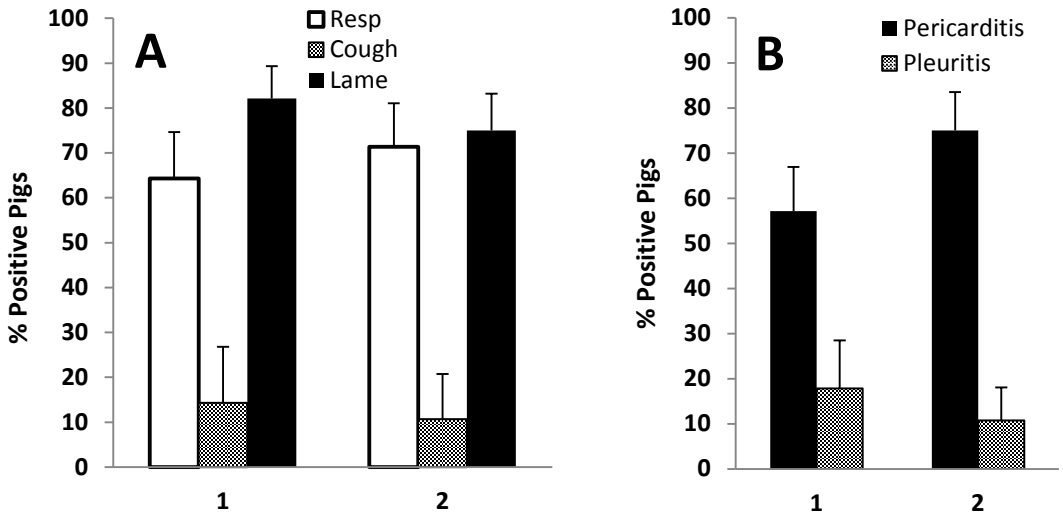

Fig. 2.3. Study 2 clinical evaluations by group. A. Assessment for abnormal respiration, coughing, and lameness. **B**. Assessment for polyserositis. Groups: 1=IP, IV, IN, 2=IP, IV. IP=intraperitoneal; IV=intravenous; IN=intranasal. All group 3 non-infected controls remained free from clinical findings and are not represented. No peritonitis was observed. Data represents the percent (%) positive animals ± standard error. There were no significant differences between groups for any category.

The average daily gains for Study 2 were calculated to be 0.56 kg/day and 0.51 kg/day for group 1 and group 2, respectively, as shown in Figure 2.4. Non-infected group 3 animals were housed separately from the challenged animals and statistical comparisons were performed for the challenged groups only.

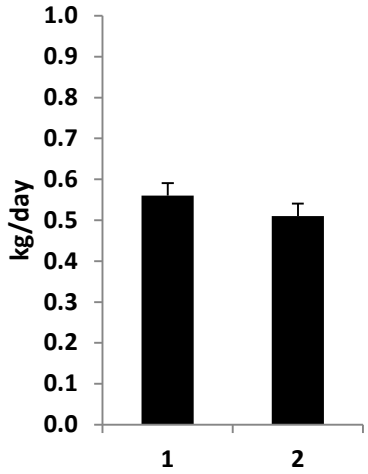

Fig. 2.4. Study 2 average daily weight gain by group. Groups: 1=IP, IV, IN, 2=IP, IV. IP=intraperitoneal; IV=intravenous; IN=intranasal. Comparisons were made between group 1 and 2 and therefore the non-infected group 3 data is not shown. Data represents the least squares mean kg/day ± standard error. There was no significant difference between groups.

Gross pathology

In Study 1, pericarditis was noted in all challenged groups with the exception of group 3. As seen in Figure 2.1B, group 2 had the highest incidence with 90% affected. Groups 4 and 5 each had 50%, while group 1 had 20% affected. Pleuritis was noted in three challenged groups with 30% of the pigs in group 2, 40% of the pigs in group 4, and 20% of the pigs in group 5 affected. Peritonitis was only noted in one animal (10%) from group 2. All of the animals in the non-challenged group 6 remained free from polyserositis.

Signs of arthritis were present in at least one animal in each challenged group. As shown in Figure 2.1A, the number of animals with at least one joint positive for arthritis were

higher in group 1 (30%), group 4 (50%) and group 5 (50%) than in group 2 (10%) and group 3 (10%). None of the group 6 non-challenged animals showed signs of arthritis in any joint.

A single mortality occurred during this study. One animal in group 2 was found dead on Day 19 and upon gross examination, was found to have pleuritis and severe pericarditis consistent with MHR infection.

The distribution of polyserositis for Study 2 can be seen in Figure 2.3B. The incidence of pericarditis was high with over half of the animals in group 1 (57.1%) and group 2 (75.0%) affected. Pleuritis was noted in 17.9% of the animals in group 1 and 10.7% of the animals in group 2. No peritonitis was observed in either group in Study 2. Non-infected group 3 animals remained free from gross lesions.

A single mortality occurred in Study 2. One animal in group 1 was found dead on Day 14. Upon necropsy, this pig was noted to have severe pericarditis and focal areas of pleuritis with areas of consolidation consistent with MHR infection.

The number of animals with at least one joint positive for signs of arthritis in Study 2 was 35.7% for group 1 and 50.0% for group 2. Figure 2.5 shows the distribution of arthritis among the eight joints examined. The tarsi were most often affected with 21.4% of the left tarsi and 28.6% of the right tarsi affected in group 1 and 35.7% of both the right and the left tarsi affected in group 2. The left elbow (14.3%), right elbow (10.7%), left stifle (10.7%), and right stifle (10.7%) of group 1 also exhibited signs of arthritis. Similarly, the left elbow (28.6%), right elbow (25%), left stifle (21.4%) and right stifle (14.3%) of group 2 were affected. The carpi for both groups showed no signs of arthritis. No signs of arthritis were noted in any joint for any of the non-infected group 3 animals.

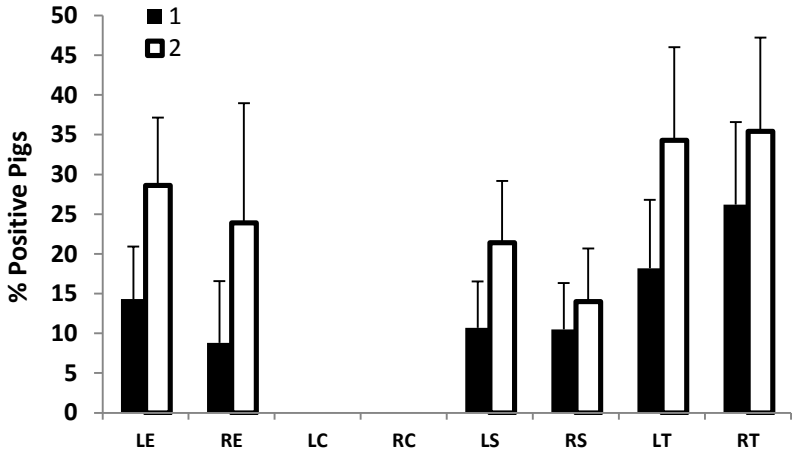

Fig. 2.5. Study 2 assessment of arthritis by joint and group. Groups: 1=IP, IV, IN, 2=IP, IV. IP=intraperitoneal; IV=intravenous; IN=intranasal; LE, RE = left and right elbow; LC, RC = left and right carpal; LS, RS = left and right stifle; LT, RT = left and right tarsal. Data represents the percent (%) animals positive for arthritis at that joint ± standard error. There were no significant differences between groups for any joint

Histopathology

Histopathology was only performed for Study 1. Samples from group 3 were free of any significant findings, while groups 2, 4 and 5 had the highest degree of microscopic lesions as shown in Table 2.2. Epicardial/pericardial adhesions, fibrosis and lymphocytic inflammation were noted in affected heart samples. All samples from non-infected group 6 showed no significant findings. Figure 2.6A shows the percentage of animals in each group receiving a score > 0 for each category. These results paralleled the gross pathological observations shown in Figure 1B.

Table 2.2. Histopathology scores for Study 1.

Group		Lung Tissue		Heart Tissue	
		Pleuritis[A]	**Infiltrates[A]**	**Inflammation[B]**	**Fibrosis[C]**
1	Affected pigs/total pigs	0/10	3/10	1/10	3/10
	Total Group Score	0	4	3	6
2	Affected pigs/total pigs	2/9	5/9	6/9	8/9
	Total Group Score	4	8	13	19
3	Affected pigs/total pigs	0/10	0/10	0/10	0/10
	Total Group Score	0	0	0	0
4	Affected pigs/total pigs	2/10	4/10	3/10	4/10
	Total Group Score	2	4	6	8
5	Affected pigs/total pigs	3/10	4/10	5/10	5/10
	Total Group Score	4	5	9	11
6	Affected pigs/total pigs	0/6	0/6	0/6	0/6
	Total Group Score	0	0	0	0

Groups: 1—IP, 2—IV, 3—IN, 4—IP, IV, IN (high dose), 5—IP, IV, IN (low dose), 6—no treatment.

A = pleuritis and peribronchiolar/septal infiltrates scored as none (0), mild (1), moderate (2), or severe (3). B = lymphocytic inflammation scored as no inflammation (0), mild accumulation of lymphocytes (1), moderate accumulation of lymphocytes (2), or severe accumulation of lymphocytes (3). C = fibrosis of the epicardium scored as no fibrosis (0), mild fibrosis (1), moderate fibrosis (2), or severe fibrosis (3).

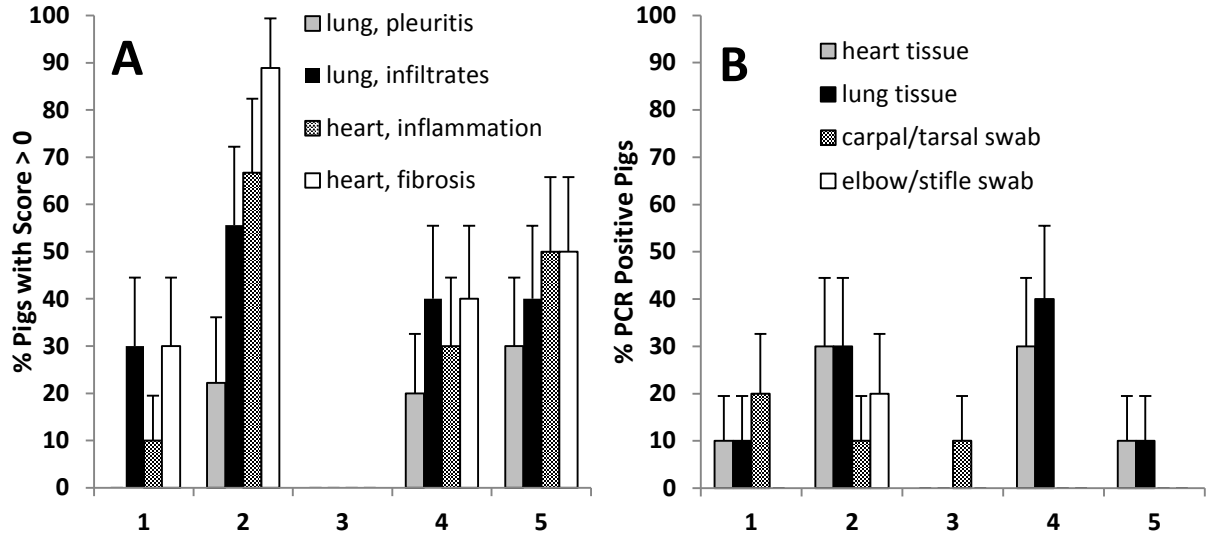

Fig. 2.6. Study 1 *in vitro* testing. A. Histological evaluations for lung (pleuritis and peribronchiolar/septal infiltrates) and heart (lymphocytic inflammation and fibrosis of the epicardium). **B**. *M. hyorhinis* PCR for heart tissue, lung tissue and joint swabs. Groups: 1=IP (intraperitoneal), 2=IV (intravenous), 3=IN (intranasal), 4=IP, IV, IN (high dose), 5=IP, IV, IN (low dose). All group 6 non-infected controls received no histological scores and were all PCR negative and are therefore not represented. Histological data represents the percent (%) animals with a score of > 0 ± standard error. PCR data represents the percent (%) animals PCR positive for the respective samples ± standard error.

PCR

For Study 1, all nasal swabs collected on D0 were negative for MHR and MHS by PCR, demonstrating a lack of colonization by either organism prior to study initiation.

Study 1 joint swabs resulted in few positive results for MHR. For the elbow-stifle swabs, 20% were positive in group 1 and 10% in both group 2 and 3; none of the group 4 or 5 samples were positive for MHR. For the carpi-tarsi swabs, 20% of the samples in group 2 were positive for MHR by PCR; all samples in group 1, 3, 4, and 5 were negative. Joint

swabs collected from non-infected group 6 were negative for MHR by PCR. All joint swabs were negative for MHS by PCR.

Fresh lung tissue from Study 1 was homogenized prior to total nucleic acid extraction and PCR testing. For groups 1-5, 10%, 20%, 0%, 40% and 10% of lung samples, respectively, were positive for MHR by PCR. Lung samples collected from non-infected group 6 were negative for MHR by PCR. All lung samples were negative for MHS by PCR.

Fresh heart tissue from Study 1 was homogenized prior to total nucleic acid extraction and PCR testing. Results for MHR PCR closely match results for the lung tissue. For groups 1-5, 10%, 20%, 0%, 30% and 10% of heart samples, respectively, were positive for MHR by PCR. Heart tissue samples collected from non-infected group 6 were negative for MHR by PCR. All heart samples were negative for MHS by PCR. See Fig. 2.6B for a summary of Study 1 MHR PCR results by group.

For Study 2, all nasal swabs collected on Day 0 were negative for MHR, MHS, and MHP by PCR, demonstrating a lack of colonization by any of these *Mycoplasma* species prior to study initiation.

Greater than half of the joint swabs collected in from both groups in Study 2 were positive for MHR by PCR as shown in Figure 2.7. For the elbow-stifle swabs, 53.4% of group 1 samples and 64.0% of the group 2 samples were MHR positive by PCR. For the carpi-tarsi swabs, 60.8% of group 1 samples and 75.4% of the group 2 samples were MHR positive by PCR. All joint swabs collected from non-infected group 3 animals were negative for MHR by PCR. All joint swabs were negative for MHS and MHP by PCR.

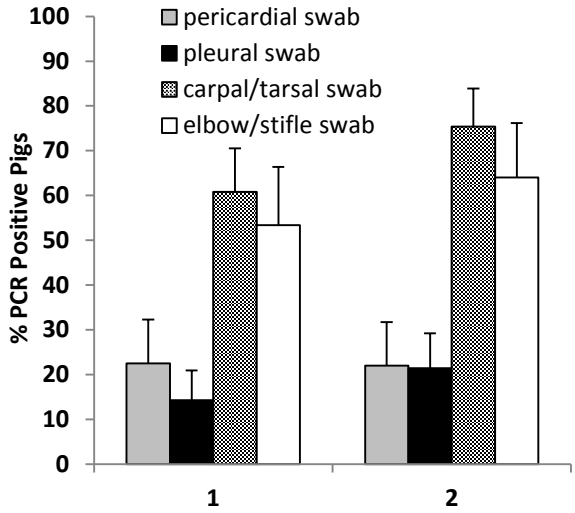

Fig. 2.7. Study 2 *M. hyorhinis* PCR for heart, lung, and joint swabs. Groups: 1=IP, IV, IN, 2=IP, IV. IP=intraperitoneal; IV=intravenous; IN=intranasal. All group 3 non-infected controls were PCR negative and are therefore not represented. Data represents the percent (%) animals PCR positive for the respective sample ± standard error. There were no significant differences between groups for any sample.

In lieu of fresh tissue collection, swabs of the pericardium and pleura were collected from animals in Study 2. As shown in Figure 2.7, 22.5% of group 1 and 22.0% of group 2 pericardial swabs were positive for MHR by PCR. For the pleural swabs, 14.3% of group 1 samples and 21.4% of group 2 samples were positive for MHR by PCR. All pericardial and pleural swabs from non-infected group 3 animals were negative for MHR by PCR. All pericardial and pleural swabs were negative for MHS and MHP by PCR.

Bacteriology

Aerobic and anaerobic culture of all necropsy samples on blood agar showed no growth of any significant bacterial pathogens (i.e. *Streptococcus suis* and *Haemophilus parasuis*).

Discussion

The challenge isolate used in our studies was found to grow to higher titers in tissue culture as compared to standard in vitro broth techniques. This is not uncommon for some strains of MHR (11). Our initial animal experiments proved this material was indeed virulent and suitable for animal challenge (data not shown).

Previous studies (6, 12, 13) have reported that a single IP administration of MHR, or combination of IP and IN on a single day, can result in polyserositis and/or lameness using traditional, broth-derived cultures of MHR. However, these studies either used extremely low group numbers (n=2) or infected piglets at ages (e.g. three weeks; three months) outside of which current literature suggests animals are most susceptible (six to seven weeks) (1). Our initial efforts in reproducing these results were mixed (data not shown). In development of this challenge model, our goal was to achieve greater than 50 % of both polyserositis and lameness in inoculated animals in order to suitably evaluate potential vaccine candidates for these clinical manifestations of MHR infection.

Treatment groups for Study 1 were confounded by housing and, therefore, a direct statistical comparison across all five groups cannot be made. However, results indicate that clinical manifestation of disease was dependent more on the route of administration than the given dose when utilizing cell-associated MHR challenge material. This is evidenced by comparing results from group 2 (IV administration) and group 3 (IN administration) which both received single inoculations at the same dose of MHR (7.96×10^8 CCU/pig). Differences in clinical outcomes differ greatly: IV administration resulted in substantial polyserositis, both grossly and microscopically, while IN administration showed no signs of

polyserositis. Additionally, a single IP administration (group 1) at twice the dose (1.59×10^9 CCU/pig) of that listed above did not induce the same level of polyserositis as the IV administration (group 2), but did result in an increased incidence of lameness compared to the other single-dose administrations (Fig. 2.1A and B). Further, pigs administered the three consecutive-day IP, IV, and IN challenge (group 4 and 5) showed a more robust disease manifestation, resulting in both polyserositis and lameness. It is unclear why the lower dose administered to group 5 (1.43×10^9 CCU/pig) resulted in greater lameness than group 4 (2.86×10^9 CCU/pig) when other results (pericarditis, arthritis, and weight gain) for these two groups were similar. We have attempted to reduce the subjectivity in lameness observations and account for incidental or transient lameness by defining a "lame" animal as one observed with lameness scores on at least two consecutive days.

It should also be noted that group 5 received a similar total dose as the single IP administration (group 1), yet group 5 had higher numbers of affected pigs for polyserositis, lameness, and arthritis as well as reduced ADG. Perhaps the additional handling time and manipulations that groups 4 and 5 incurred stressed these animals more than the others, increasing anxiety as well as the susceptibility to infection. We did not control for the possibility of this effect in either study.

Study 1 provided sufficient proof of concept data to further optimize the multiple route, consecutive-day approach for the MHR challenge model. Mock infections were performed using antigen-free cultures and showed that clinical manifestations were *Mycoplasma*-dependent (data not shown). Study 2 was designed to evaluate the necessity of the third-day, IN challenge in the model as Study 1 indicated minimal success with IN

administration alone. Study 2 also terminated at 28 days post-challenge (dpc), seven days later than Study 1. This extension was included to determine if mortalities would increase given the severity of pericarditis observed in the model. No increase in mortality was observed, however, two additional group 2 animals were scored as lame due to the seven day extension. Treatment groups were co-mingled in this study and, therefore, direct comparisons between groups were possible. Results from Study 2 are clear: there were no significant differences noted between animals administered three-consecutive-day inoculation (IP, IV, and IN) and animals administered two-consecutive-day inoculation (IP and IV only) for any parameter measured. The IN administration can, consequently, be omitted from the challenge model protocol without negatively affecting clinical outcomes of MHR infection. This omission will reduce time and resources in both the laboratory, for producing sufficient quantity of challenge material, and in the animal facilities, for time allotted to animal manipulations.

The differences in outcomes between the multiple route, consecutive-day challenge and the single route, single-day challenges may be due to a weaker overall inflammatory response in the later and therefore resulting in less joint involvement; whereas the former may allow for sufficient and chronic inflammatory response, leading to polyarthritis (14). Further, it is possible that the consecutive-day approach allows for antigen over-abundance and accumulation of small immune complexes leading to a Type III hypersensitivity reaction (15) or autoimmunity (16).

The natural route of infection and the pathogenesis for MHR are currently unknown (1, 2). Our results indicate that systemic infection via the circulatory and/or lymphatic

system is highly likely. Although the route of initial entry into the host still remains unclear, MHR colonizes the upper respiratory tract (2), and it is very likely the organism enters the host via this route. Our results for IN inoculation, though, provide little data to support this hypothesis. Perhaps given sufficient time (> 21 days post challenge) animals administered MHR by IN inoculation might indeed succumb. It is also possible that environmental MHR is simply more efficient with colonization and infection than in vitro cultures which make feasible the upper respiratory route as a means of entry. Alternatively, fighting is common among wean-to-finish pigs and perhaps biting provides the mechanism of entry, creating an opening for which MHR can be transmitted from a colonized animal directly to the bloodstream of another.

Conclusion

We have shown that the optimized, consecutive-day inoculation using cell-associated MHR material was successful in achieving > 50 % affected animals for both lameness and pericarditis. The model can therefore be defined as administration of cell-associated MHR to seven week old CDCD pigs at 20 mL/IP and 10 mL/IV on the first and second day of challenge, respectively, for a total recommended dose of $1.5 \times 10^9 \pm 0.5$ CCU per pig.

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CHAPTER 3. AGE SUSCEPTIBILITY OF CAESARIAN DERIVED COLOSTRUM DEPRIVED PIGS TO *MYCOPLASMA HYORHINIS* CHALLENGE

A paper prepared for submission to a peered reviewed journal

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Abstract

Mycoplasma hyorhinis (MHR) is a major cause of lameness, arthritis, and polyserositis among grower pigs. Reduced performance and culling due to MHR infection result in economic losses in swine production.

We have developed an MHR challenge model in seven week old CDCD pigs using cell-associated MHR which results in both severe pericarditis and lameness. In this study we sequentially challenged CDCD pigs at seven, ten, thirteen, and sixteen weeks of age with our cell-associated challenge material. Lameness was observed in over 60 % of the animals in the first three age groups but fell to 33 % in the oldest age group. The number of animals with arthritis fell from 100 % at seven weeks, to 56 % at ten weeks and approximately 25 % at both thirteen and sixteen weeks of age. Pericarditis was observed in 87 % of the seven week challenge group, 28 % in the ten week challenge group, 8 % in the thirteen week challenge group and 4 % in the sixteen week challenge group. All challenged groups showed an average daily gain (ADG) of at least 0.6 lbs/day less than the age-matched non-challenged

control groups, with the largest margin noted at thirteen weeks of age at a 1.2 lbs/day difference.

Results of this study show that animals were susceptible to MHR-associated lameness through sixteen weeks of age while susceptibility to MHR-associated polyserositis appeared to peak at seven weeks of age.

Introduction

Mycoplasma hyorhinis (MHR) causes disease in three- to ten-week old animals but most commonly causes polyserositis and polyarthritis in pigs around seven weeks of age (1, 2). Economic losses due to MHR can be attributed to reduced performance (weight gain and feed conversion) and culling due to lameness (3). There are currently no licensed, commercially available vaccines for MHR.

We have previously established a robust experimental infection protocol for MHR in seven-week old caesarian derived-colostrum deprived (CDCD) pigs which results in severe pericarditis and lameness (submitted). Some literature has suggested an age limitation to the susceptibility of MHR (4) although eight-weeks of age was the limit of testing. In this study, we evaluated our MHR challenge model using cell associated challenge material in the same litters of animals at seven, ten, thirteen, and sixteen weeks of age. The results demonstrate the age of greatest susceptibility and thus, can define the preferred duration of immunity for any potential vaccine as well as provide guidance for prophylactic antibiotics to minimize their use.

Materials and Methods

Challenge material

The MHR challenge isolate originated from the joint fluid of a pig displaying clinical signs of mycoplasma infection. After initial culturing in modified Friis media (5), the infection dose of MHR was cultured on confluent monolayers of MDCK (Madin-Darby canine kidney) cells. Cell maintenance media was minimum essential media (MEM; Gibco/Thermo Fisher Scientific Inc., Waltham, MA) supplemented with 10 % fetal bovine serum (FBS; SAFC, St. Louis, MO). Growth media was removed prior to infection and Mycoplasmas were then added to the tissue culture at approximately 10 % (v/v) of the total final volume. Cells with mycoplasma were allowed to incubate at 37° C for approximately two hours before adding pre-warmed media consisting of MEM supplemented with 2 % FBS. After the presence of cytopathic effect (CPE) was noted, generally occurring around 4-7 days post-infection, the culture was harvested by briefly shaking the flasks and collecting the supernatant. The MHR culture was then combined with 10 % (v/v) sterile glycerol, dispensed into working volumes and stored at < -60° C until challenge. Quantification of the challenge doses was performed by color changing units (CCU).

No freezing step was used prior to harvest as described previously (submitted). This preparation greatly reduced the amount of cellular debris in the challenge material which, in turn, reduced incidences of anaphylaxis post-intravenous challenge.

Animal information

Commercial cross breed CDCD piglets, both gilts and in-tact barrows, were acquired from a Class A dealer. Nasal swabs (eSwab™, Copan; Murrieta, CA) were collected prior to

challenge and were negative for MHR, *M. hyopneumoniae* (MHP) and *M. hyosynoviae* (MHS) as determined by real-time PCR analysis. Sera were also collected prior to challenge and were free from MHP antibodies by ELISA (IDEXX, Westbrook, ME). Animals were seven weeks ± five days of age at the time of study initiation and were deemed to be in good health and nutritional status. After arrival to the study site, no biologicals or pharmaceuticals other than the challenge material were administered to the test animals. Non-medicated feed was used at rations appropriate for the age, condition, and species of test animal. Water was provided *ad libitum*. Housing was a BSL-2 USDA inspected facility and the study was conducted following the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (6).

Pigs were blocked by litter and randomly divided into four groups of 25 animals each for experimental challenge, labeled groups 1-4. Four groups of five pigs each were used for non-challenged control animals, labeled groups 5-8. Animals were housed in four rooms with two pens per room. Pens were a raised deck with metal slatted flooring and metal gating for sides. Pigs in Group 1 and 4 were housed in separate rooms. Pigs in Group 2 and 3 were housed together until challenge for Group 2, at which time animals in Group 2 were segregated into their own room. Pigs in Group 4 were housed in two rooms at the time of challenge due to size and space requirements. Non-challenged animals in Group 5-8 were housed independently of the challenged animals and co-mingled together in one room in two pens.

Experimental inoculation

Pigs in Group 1 were inoculated with MHR at 20 mL/intraperitoneal injection (IP) on Day 0, 10 mL/intravenous injection (IV) on Day 1, and 10 mL/intranasal application (IN; 5 mL/nostril) on Day 2, for a total dose of 5.73×10^9 CCU/pig. Pigs in Group 2, 3 and 4 were inoculated with MHR in identical fashion beginning on Day 21 (2.55×10^9 CCU/pig), Day 42 (3.72×10^9 CCU/pig), and Day 63 (4.26×10^9 CCU/pig), respectively (see Table S17 in Appendix B). The MHR inoculum was titrated by CCU using five replicates per assay, in triplicate, on each challenge day. The dose for any given day was recorded as the mean of the triplicate assays and the total applied dose was the summation of the three mean titers. All titers for the first, second and third day of challenge for any given group, as well as across groups on any given day, resulted in a coefficient of variation (CV) of less than 5%. The CV for all 36 titrations was less than 5%.

Clinical observations

Pigs were observed daily for lameness during their respective challenge periods, from the first challenge day (0 dpc) through 21 days post challenge (21 dpc). Lameness was scored 0-5 according to the system outlined in Table 3.1. A pig was considered lame if it received a score of

≥ 1 on any two (or more) consecutive days. Weight measurements were taken just prior to challenge on 0 dpc and just prior to euthanasia on 21 dpc. At study termination, all pigs were anesthetized and then euthanized by electrocution. The experimental schedules of events are summarized in Table S18 in Appendix B.

Table 3.1. Scoring system for lameness.

Score	Description
0 =	**Normal**—no visible lameness.
1 =	**Mild**—not constantly lame when walking, walks at a normal speed, is weight bearing while walking and standing, lameness is indicated by intermittent reduced weight bearing on one limb or shortening of the stride.
2 =	**Moderate**—constant and observed throughout every step at a walking pace, bearing some weight on the leg at a walk and standing, but short-striding one or more legs while walking, walks at a normal speed.
3 =	**Moderately Severe**—puts no weight on the leg the first few steps after standing, constant, obvious lameness while at a walking pace, putting very little weight on the leg at a walk or while standing, requires the pig to slow its speed of walking.
4 =	**Severe**—will stand (may require assistance) for at least 3 minutes, non-weight bearing on one or more legs at walk or standing, still able to three-legged walk.
5 =	**Recumbent**—will not stand even with assistance.

Gross pathological examination and sample collection

Following euthanasia, a gross pathological examination was performed. The thoracic and abdominal cavities were opened and examined for evidence of polyserositis. Pericardial swabs (eSwab™, Copan; Murrieta, CA) were taken from all pigs. Pleural swabs were taken from only those animals exhibiting pleuritis. Serosal swabs were taken from only those animals exhibiting peritonitis. All swabs were tested for MHR, MHS and MHP by real-time

PCR. The elbows, stifles, carpi and tarsi were opened and examined for signs of arthritis including, but not limited to, abnormal and/or excessive synovial fluid, abnormal synovial membranes, and abnormal articular surfaces. For each pig, a single swab (eSwab™, Copan; Murrieta, CA) was used to sample the articular surfaces of both elbows and both stifles. This process was repeated using a new, single swab for all four carpi and tarsi. All joint swabs were tested for MHR, MHS, and MHP by real-time PCR.

Real-time PCR

Total nucleic acid extractions and real-time PCR were performed as described previously (Chapter 2, page 25). The method of detection by real-time PCR for MHP has been previously described (7).

Statistical analyses

Statistical summaries were conducted using SAS Version 9.4. Treatment was confounded by housing and results were therefore summarized by treatment group. Individual average daily gain during the 21 day challenged period was calculated by subtracting the 0 dpc weight from the 21 dpc weight and dividing by 21. Average daily gain for each treatment group was summarized using the MEANS procedure of SAS. Means, standard errors, and 95% confidence intervals (CI's) for each treatment were determined (Table S19). The presence of pericarditis, pleuritis, and peritonitis, as well as PCR swab results were each summarized by treatment group using the FREQ procedure of SAS. The proportion positive, standard error, and Clopper-Pearson Exact 95% CI's were determined for each treatment group (Table S20 and S21). The percent of joints with arthritis for each animal was summarized by group using the MEANS procedure of SAS. Means, standard

errors, and 95% CI's for each treatment were determined (Table S22). In addition, the percent of animals with at least one affected joint was summarized using the FREQ procedure of SAS. The proportion positive, standard error, and Clopper-Pearson Exact 95% CI's were determined for each treatment group (Table S22). The presence or absence of lameness during the 21 day challenge period was determined and summarized. The proportion of lame animals within each treatment group was reported with the standard error and Clopper-Pearson Exact 95% CI's using the FREQ procedure of SAS (Table S23).

Results

Clinical observations

Lameness was observed in all challenged groups 1-4 (Fig. 3.1). Group 1, 2, and 3 all resulted in over half of the animals lame in each group with 65.2 %, 68.0 %, and 68.0 % affected animals, respectively. Fewer pigs were affected in group 4 with 33.3 % scored as lame.

None of the animals in each of the non-challenged groups 5 and 7 were scored as lame. One animal in the non-challenged group 6 received a lameness score of "1" from 5 dpc (Day 26) through 10 dpc (Day 31). Two animals in the non-challenged group 8 received lameness scores. One pig received a score of "1" on the last three observation days, 19 dpc (Day 82) through 21 dpc (Day 84). The other pig was noted with lameness very early in the observation period receiving a score of "2" on 1 dpc (Day 64), scores of "1" on 4 and 5 dpc (Day 67 and 68), scores of "2" on 6 and 7 dpc (Day 69 and 70), and scores of "1" on each of the remaining observation days, 8 dpc (Day 71) through 21 dpc (Day 84).

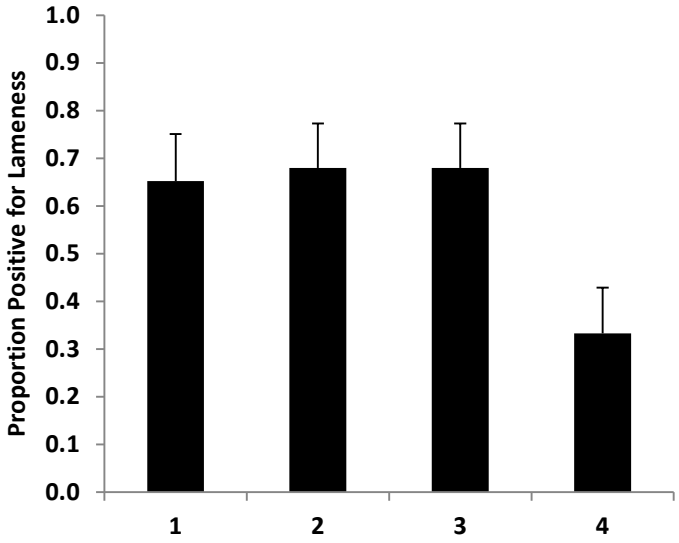

Fig. 3.1. Assessment of lameness. Groups: **1** = 7 weeks of age; **2** = 10 weeks of age; **3** = 13 weeks of age; **4** = 16 weeks of age. Data are for the MHR challenged groups only and represent the proportion positive animals per group ± standard error.

There was little difference between the mean weight for challenged and non-challenged animals on 0 dpc as seen in Table 3.2. At necropsy (21 dpc), however, there was a large discrepancy between the mean weight of animals challenged with MHR and those not receiving challenge. For the seven-week age groups, non-challenged group 5 animals weighed an average of 18.0 lbs. more than group 1. For the ten-week age groups, non-challenged group 6 animals weighed an average of 17.7 lbs. more than group 2. For the thirteen-week age groups, non-challenged group 7 weighed an average of 30.2 lbs. more than group 3. For the sixteen-week age groups, non-challenged group 8 weighed an average of 20.7 lbs. more than group 4.

Table 3.2. Mean weight (lbs) on first and last day of challenge.

Treatment Group*	n	0 dpc Mean Weight (lbs) ± Standard Error	0 dpc Difference, Challenge – Non-challenge (lbs)	21 dpc Mean Weight (lbs) ± Standard Error	21 dpc Difference, Challenge – Non-challenge (lbs)
1	23	22.0 ± 0.67	-0.8	32.0 ± 1.36	-18.0
5	5	22.8 ± 0.80		50.0 ± 1.65	
2	25	50.8 ± 1.02	-0.2	69.6 ± 1.76	-17.7
6	5	51.0 ± 4.21		87.3 ± 5.64	
3	25	93.5 ± 1.87	-5.2	114.9 ± 2.44	-30.2
7	5	98.7 ± 2.98		145.1 ± 5.79	
4	24	125.7 ± 2.24	-7.0	160.3 ± 2.88	-20.7
8	5	132.7 ± 5.45		181.0 ± 6.65	

*__1 (Challenge) and 5 (Non-challenge)__ = 7 weeks of age (0 dpc = Day 0);
__2 (Challenge) and 6 (Non-challenge)__ = 10 weeks of age (0 dpc = Day 21);
__3 (Challenge) and 7 (Non-challenge)__ = 13 weeks of age (0 dpc = Day 42);
__4 (Challenge) and 8 (Non-challenge)__ = 16 weeks of age (0 dpc = Day 63).
dpc = days post challenge

The average daily gain (ADG) was also calculated for all groups (Fig 3.2). All non-challenged animals (groups 5-8) gained an average of ≥ 1.3 lbs. per day with group 7 and 8 averaging a gain of ≥ 2.2 lbs. per day. In contrast, three of the four challenged groups (groups 1-3) gained on average ≤ 1.0 lb. per day with group 4 having an average gain of 1.7 lbs. per day.

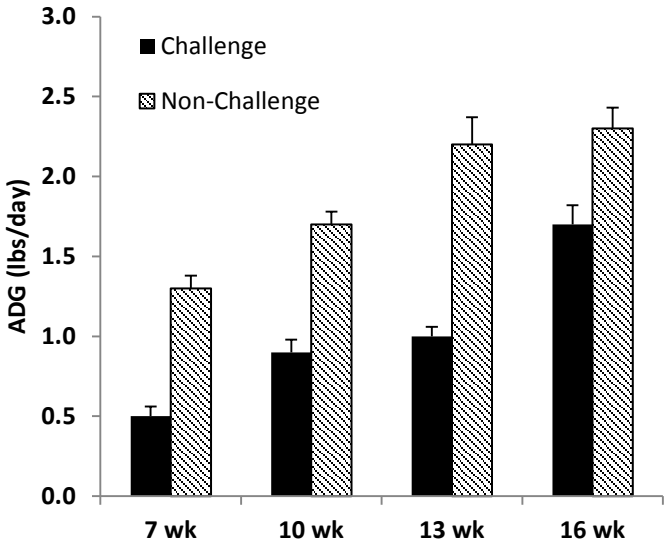

Fig. 3.2. Average daily weight gain. Groups: **7 wk** = seven week old animals, group 1 (challenge) and group 5 (non-challenge); **10 wk** = ten week old animals, group 2 (challenge) and group 6 (non-challenge); **13 wk** = thirteen week old animals, group 3 (challenge) and group 7 (non-challenge); **16 wk** = sixteen week old animals, group 4 (challenge) and group 8 (non-challenge). Data represent the mean lbs/day ± standard error. ADG = average daily gain.

One animal in group 1 was excluded (euthanized) from the study. This animal was observed to be lame at the time of study initiation on 0 dpc. No data from this animal was included in the analyses.

Mortalities, although few, were noted in all challenged groups. Results from all of the following animals were included in the analyses. One pig in group 1 was found dead on 9 dpc, and upon necropsy was noted to have an active gastric ulcer at the pars esophagea. This animal was also noted to have mild pericarditis, peritonitis, and excess synovial fluid in both rear tarsi. With the exception of the ulcer, these clinical signs are consistent with MHR infection; however, death was most likely attributable to ulceration of the pars esophagea (UPE).

One animal in group 2 was found dead on 13 dpc and upon necropsy was noted to have a pale carcass and a large, active gastric ulcer at the pars esophagea. This animal was also noted to have excess synovial fluid in the left elbow. Death was most likely attributable to UPE.

Two animals in group 3 did not make it to the end of the challenge period. One animal was euthanized for humane reasons on 11 dpc due to severe lameness (recumbent). Both stifles had excess synovial fluid. The clinical signs of disease were consistent with MHR and mortality was most likely due to virulent challenge. Another animal in group 3 was found dead on 19 dpc; however, no gross lesions were observed at necropsy and the cause of death could not be determined.

One animal in group 4 was found dead on 18 dpc. No gross lesions were noted, although excess synovial fluid was noted in the tarsi and both stifles. Lung tissue was submitted to Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL) for further examination. Hematoxylin and eosin (H/E) staining of fixed lung showed moderate interlobular and perivascular edema. Multifocal alveoli contained edema fluid with scant fibrin. Routine culture of fresh lung tissue showed no significant bacterial growth. The cause of death could not be determined.

Gross pathology

The incidence of polyserositis can be seen in Figure 3.3. Pericarditis was observed in 87.0 % of the animals in group 1. The number of affected pigs declined in subsequent challenges with 28.0 % in group 2, 8.0 % in group 3, and 4.2 % in group 4. All non-challenged animals in groups 5-8 remained free from pericarditis.

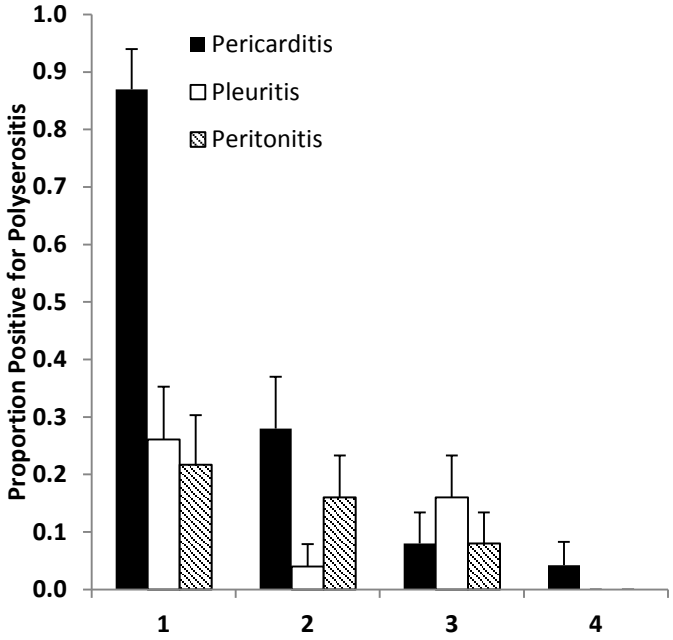

Fig. 3.3. Gross assessment for polyserositis. Groups: **1** = 7 weeks of age; **2** = 10 weeks of age; **3** = 13 weeks of age; **4** = 16 weeks of age. Data are for the MHR challenged groups only and represents the proportion positive animals ± standard error.

Pleuritis was observed in 26.1 % of the animals in group 1, 4.0 % of the animals in group 2, and 16.0 % of the animals in group 3. None of the animals in group 4 were affected with pleuritis. All non-challenged animals in groups 5-8 remained free from pleuritis.

Peritonitis was noted in 21.7 % of the animals in group 1, 16.0 % of the animals in group 2, and 8.0 % of the animals in group 3. None of the animals in group 4 were affected with peritonitis. All non-challenged animals in groups 5-8 remained free from peritonitis.

The number of pigs per group with at least one joint exhibiting signs of arthritis decreased as the age of the animals increased (Fig. 3.4A). All of the animals (100 %) in group 1 showed at least one arthritic joint. The incidence of arthritis fell in subsequent challenges with 56.0 % of the animals affected in group 2, 24.0 % of the animals affected in

group 3, and 25.0 % of the animals affected in group 4. All non-challenged animals in groups 5-8 showed no signs of arthritis in any joints examined.

Further, the number of affected joints per pig was analyzed in the challenged groups (Fig. 3.4B). The total number of joints affected per animal decreased with the increase of age at challenge. Of the eight joints examined, the average proportion of positive joints was 46.2 % for group 1, 17.5 % for group 2, 4.0 % for group 3, and 8.9 % for group 4.

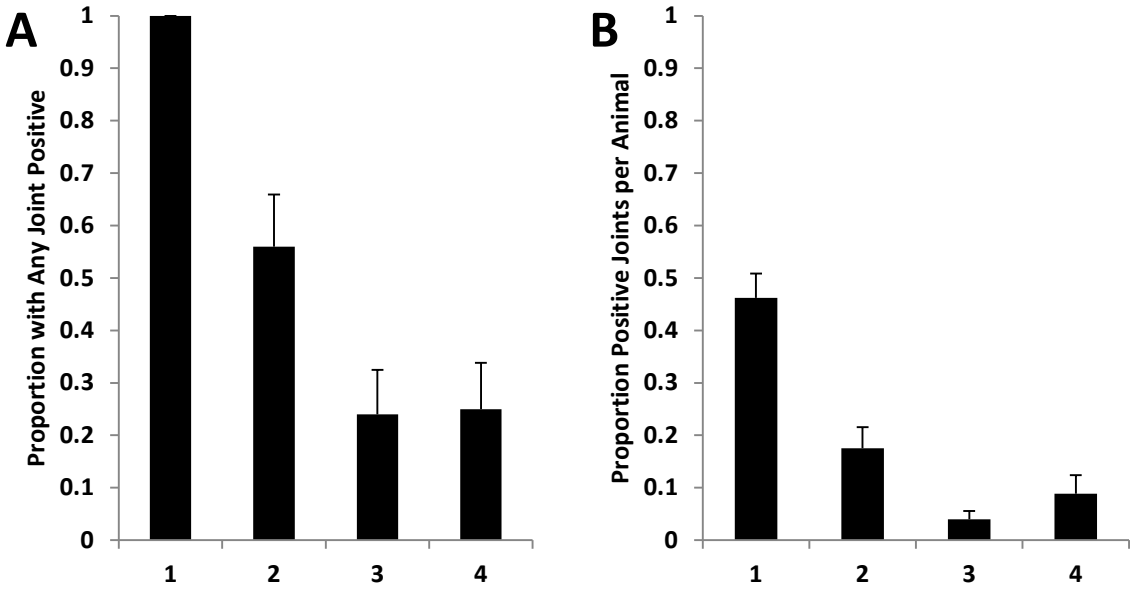

Fig. 3.4. Assessment for arthritis. A. Proportion of animals per group with at least one joint showing signs of arthritis. Data are for MHR challenged groups only and represent the proportion of positive animals ± standard error. **B**. Proportion of affected joints per animal in each group. Data are for MHR challenged groups only and represent the proportion positive joints ± standard error. Groups: **1** = 7 weeks of age; **2** = 10 weeks of age; **3** = 13 weeks of age; **4** = 16 weeks of age.

Real-time PCR

Nasal swabs collected one day prior to each challenge were all negative for MHR, MHS, and MHP by PCR, demonstrating a lack of colonization by any of these Mycoplasma species prior to study initiation.

Fewer than 50 % of all the serosal swabs (Fig. 3.5A) were positive for MHR by PCR. For pericardial swabs, 26.1 % of the samples from group 1, 4.0 % of the samples from group 2, and 8.3 % of the samples from group 4 were positive for MHR by PCR. All pericardial swabs from group 3 were negative for MHR by PCR. All pericardial swabs from non-challenged animals in groups 5-8 were negative for MHR by PCR. All pericardial swabs collected were negative for MHP and MHS by PCR.

Pleural swabs were only taken from those animals exhibiting gross pleuritis. Of the six pleural swabs collected from group 1, two were positive for MHR by PCR. Pleural swabs collected from group 2 and group 3 were all negative for MHR by PCR. No pleural swabs were collected from group 4. No pleural swabs were collected from the non-challenged animals in groups 5-8. All pleural swabs collected were negative for MHP and MHS by PCR.

Peritoneal swabs were only collected from those animals exhibiting gross peritonitis. Of the five samples collected from group 1, one was positive for MHR by PCR. Peritoneal swabs collected from group 2 and group 3 were all negative for MHR by PCR. No peritoneal swabs were collected from group 4. No peritoneal swabs were collected from non-challenged animals in groups 5-8. All peritoneal swabs collected were negative for MHP and MHS by PCR.

A greater number of MHR PCR positive results were detected from the joint swabs (Fig. 3.5B) than with serosal swabs. Elbow-stifle swabs collected from group 1 resulted in 82.6 % samples positive for MHR by PCR. There were fewer MHR positive results in the subsequent challenges, with 36.0 % in group 2, 32.0 % in group 3, and 16.7 % in group 4 positive by PCR. All elbow-stifle swabs from non-challenged animals in groups 5-8 were negative for MHR by PCR. All elbow-stifle swabs collected were negative for MHP and MHS by PCR.

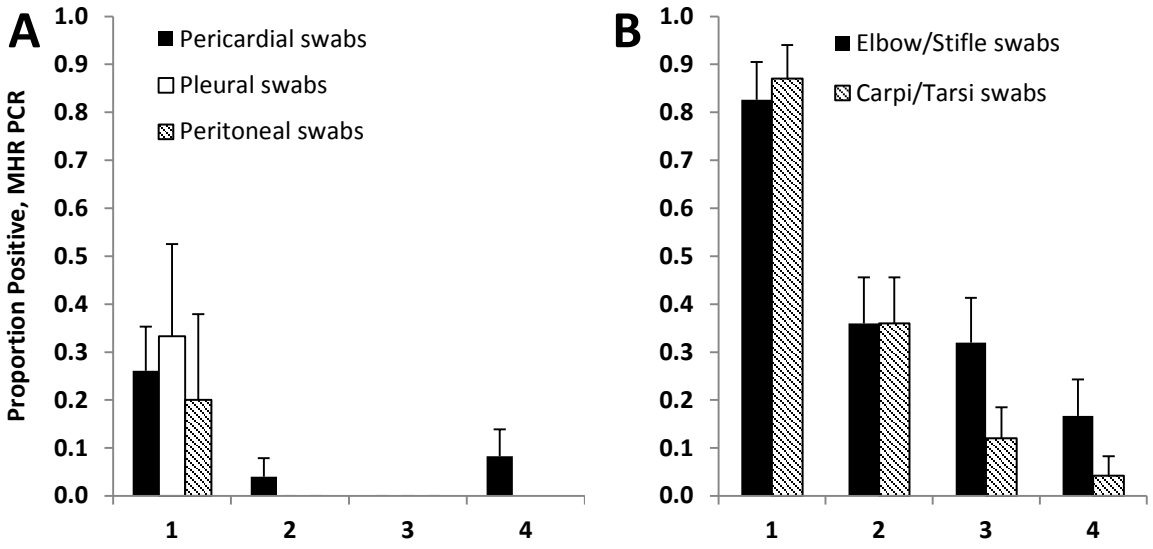

Fig. 3.5. *M. hyorhinis* PCR for necropsy swabs. A. Serosal swabs. Pericardial swabs were collected from all animals. Pleural and peritoneal swabs were collected only from those animals exhibiting gross lesions. Data are for MHR challenged groups and represent the proportion of positive samples ± standard error. **B**. Joint swabs. A single swab was used to sample surfaces of both the elbows and stifles. A single swab was used to sample surfaces of both carpi and tarsi. Data are for MHR challenged groups and represents the proportion of positive samples ± standard error. Groups: **1** = 7 weeks of age; **2** = 10 weeks of age; **3** = 13 weeks of age; **4** = 16 weeks of age.

Carpal-tarsal swabs followed a similar trend to the elbow-stifle swab PCR results. Group 1 had 87.0 % samples positive for MHR. There were fewer positive results in the subsequent challenges, with 36.0 % in group 2, 12.0 % in group 3, and 4.2 % in group 4 positive for MHR by PCR. All carpal-tarsal swabs from non-challenged animals in group 5-8 were negative for MHR by PCR. All carpal-tarsal swabs collected were negative for MHP and MHS by PCR.

Discussion

Prior research suggests piglets of six to seven weeks of age are most susceptible to natural infection by MHR (2, 8, 9). Our previous studies using cell associated challenge material support these findings (submitted). The goal of this study was to evaluate the effectiveness of our MHR challenge model and cell associated challenge material to cause disease in animals older than seven weeks of age.

The use of CDCD animals in this model makes it extremely difficult to procure the appropriate number of animals at the correct ages to challenge all groups simultaneously. Even if such requirements could be fulfilled, the size differences between the age groups tested would make it impossible to co-mingle all challenge groups as the larger animals would simply out-compete the smaller ones. With housing confounding treatment, a direct statistical comparison between groups 1-4 is not possible. However, we attempted to minimize any differences in the protocol between these groups to allow for a better understanding of how MHR affects the same litters within a herd following infection at sequential ages. This included utilization of the same stock of MHR challenge material and the same sample collection, husbandry and housing protocols.

Group 1 animals were seven weeks of age at the time of inoculation and represented the “positive control” group based on previous reports mentioned above. This group of animals confirmed the susceptibility of these litters to MHR infection as well as the effectiveness of the challenge material used throughout the study. Results for group 1 were similar to those observed in our previous challenge model evaluations with high incidences of pericarditis, lameness, and arthritis. No animals in the seven-week non-challenged group 5 showed any signs of MHR-associated disease (e.g. pericarditis, lameness, and arthritis). Further, the ADG for group 1 was approximately half of that observed for group 5. Non-challenged animals were housed separately from their age-matched, challenged counterparts to maintain biosecurity. With housing confounding treatment, a direct statistical comparison between these groups is not possible. However, results from group 1 confirm effectiveness of the challenge model in these litters of animals.

Group 2, 3, and 4 were challenged in three-week intervals at ten, thirteen, and sixteen weeks of age, respectively. The incidence of pericarditis was greatly reduced from group 1 (87 %) to group 2 (28 %) and continued to decrease in group 3 (8 %) and group 4 (4 %). Thus, protection from polyserositis by any potential vaccine candidate should extend through seven weeks at a minimum to limit the most severe pericarditis noted. Discriminate antibiotic use can also be recommended as mycoplasma-effective drugs (i.e. lincomycin) may not be necessary in older animals still exhibiting severe polyserositis if the prescriber suspected MHR as the root cause.

Lameness in group 2 and 3 (68 %) was similar to group 1 (65 %), while group 4 had a reduced incidence of lameness (33 %). Arthritis also decreased with age, from 100 %

affected animals in group 1 to 25 % in group 4. Although 1/3 of the animals at 16 weeks of age still exhibited lameness, the downward trend of lameness observations, arthritis and PCR detection indicate that vaccine protection need only carry through 10 to 13 weeks of age to limit the most severe lameness and arthritis.

It should be noted that lameness was observed in three non-challenged animals. One animal in group 6 (ten week age group) was scored as lame on six consecutive days (5 dpc through 10 dpc). Two animals in group 8 (sixteen week age group) were scored as lame. One animal was scored lame on the second day of observations and then intermittently throughout the remainder of the observation period, while the other animal was scored lame on the last three days of the observation period. The lack of any gross lesions (polyserositis and arthritis), as observed in the challenged groups, as well as the lack of any MHR PCR positive samples for these animals supports the cause of the observed lameness as non-mycoplasma related. These results do not invalidate results obtained from the challenged animals during this time period. It does, however, demonstrate that lameness alone is not necessarily an indicator of MHR infection.

The low number of MHR PCR positive pericardial swabs is similar to findings in our previous studies (data not shown). It is possible this is due to assay sensitivity; however, the recovery of MHR positive samples in the joint swabs suggest otherwise. Likely, these results could be attributable to the timing of the sample collection. Swabs are not collected until 21 days post-challenge in an effort to allow sufficient time for clinical observations of lameness to manifest. Therefore, it is likely that MHR had been cleared form the heart (and lungs) by the time of necropsy. The high number of MHR positive joint swabs may also be due to the

progression of MHR infection, indicating that the heart is affected earlier and the joints later in the course of disease. The pathogenesis of MHR has not been definitively defined and a time-course sample collection study design would be required to confirm this hypothesis.

The clinical observations of pericarditis, lameness, arthritis, and weight gain show that the severity of MHR infection lessened at each subsequent challenge from the seven week of age time-point. We have not tested our challenge model in animals younger than six weeks of age, but it may be inferred that younger animals are equally susceptible. It is possible that the older animals may be able to provide a more substantial immune response, lessening the systemic effects (polyserositis), but still resulting in chronic inflammatory arthritis as described by Cole and Cassell (10). Talker *et al*. (11) demonstrated an increase in numbers of various T cell subsets from birth through six months of age, and in particular a strong increase in γδ T cells from weaning through approximately 19 weeks of age. Perhaps the numbers of effector cells are insufficient to combat MHR until after two months of age. Although MHR was still able to cause disease in some animals, by sixteen weeks of age, signs of infection were modest.

Conclusion

To the best of our knowledge, this is the first study to evaluate the progression of MHR infection in animals of the same litters, inoculated sequentially at seven, ten, thirteen and sixteen weeks of age. Rates of polyserositis were greatly reduced in the later three groups. Lameness was equivalent among the first three age groups and lessened at sixteen weeks of age, while incidence of arthritis was reduced in the three oldest groups of animals. For all age groups, the ADG was ≥ 0.6 lbs./day below that of non-challenged animals.

MHR greatly affects animals at seven weeks of age with severity lessening through at least sixteen weeks of age.

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CHAPTER 4. EFFICACY OF AN INACTIVATED *MYCOPLASMA HYORHINIS* VACCINE IN PIGS

A paper prepared for submission to a peered reviewed journal

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Abstract

Lameness and polyserositis in pigs caused by *Mycoplasma hyorhinis* (MHR) are generally treated with antibiotics and may require multiple doses. The costs of these antibiotics combined with economic losses from culling and reduced feed conversion due to lameness are hardships to the swine producer. In this study we have demonstrated efficacy of an inactivated MHR vaccine administered to three-week old caesarian-derived colostrum-deprived (CDCD) piglets. Three doses of vaccine (high, medium, and low) were evaluated and compared to a placebo control. MHR challenge occurred three weeks after vaccination. Pigs were observed for lameness and respiratory distress for three weeks following challenge. Pigs were then euthanized and a gross pathological evaluation for polyserositis and arthritis was performed. A minimum immunizing dose of vaccine was defined as containing at least 7.41×10^7 CCU of MHR per 2.0 mL dose as represented by the medium dose vaccine. This vaccine provided significant reductions of lameness and pericarditis with preventive fractions of 0.76 (95% CI [0.26, 0.92]) and 0.58 (95% CI [0.31, 0.74]),

respectively, compared to the placebo control group. A significant increase in post-challenge weight gain ($p < 0.0001$) was also achieved with this vaccine, with an average daily gain (ADG) of 0.92 lbs/day compared to 0.57 lbs/day in the placebo group.

Introduction

Among the species of mycoplasma which are pathogenic in swine, *Mycoplasma hyorhinis* (MHR) has the most diverse range of clinical effects. As with *Mycoplasma hyopneumoniae* (MHP) and *Mycoplasma hyosynoviae* (MHS), MHR colonizes the upper respiratory tract and can easily be recovered from the nasal cavity and tonsils of infected pigs (1, 2). MHR-associated disease occurs in the lungs and joints where the bacteria cause pneumonia and polyarthritis, respectively (3-6). It is also a common cause of polyserositis (3, 5, 6). In particular, we have noted severe pericarditis in experimentally infected animals (B. T. Martinson, F. C. Minion, D. M. Jordan, submitted for publication). MHR has also been implicated in cases of eustachitis and otitis (3, 7).

The most severe MHR infections, in particular polyserositis and lameness, lead to reductions in weight gain and feed conversion as well as culling of lame animals which result in economic losses to producers (8). As no licensed commercial vaccine is currently available, treatment of MHR has typically been with prophylactic and therapeutic antibiotics.

In this study, we evaluated three different doses of an inactivated MHR vaccine in caesarian-derived colostrum-deprived (CDCD) piglets at three weeks of age for protection against MHR-associated pericarditis and lameness. We determined the minimum immunizing dose of this vaccine, demonstrated significant reductions for both of the primary parameters measured, and achieved significant increases in weight gain compared to a placebo control

group. Results indicate the use of such a vaccine would decrease production losses and the need of antibiotics for treatment of MHR-associated disease.

Materials and Methods

Experimental design

In this randomized complete block design, 120 piglets were blocked by litter and enrolled into one of four treatment groups (28 animals per group). Animals received either a placebo control product (**CTRL**) or one of three doses of our experimental MHR vaccine (**HIGH**, **MED**, and **LOW**). A fifth group of eight animals received no treatment (**NTX**) throughout the study, acting as environmental negative controls. Twenty-two days after vaccination, all animals except NTX were challenged with MHR. Animals were observed daily post-challenge for signs of respiratory distress, coughing, lameness and well-being. Twenty-one days after challenge, all animals were euthanized and a gross pathological examination was performed. The study was performed following the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (9) in BSL-2 USDA inspected facilities.

Animals

CDCD piglets were purchased from Struve Labs International (SLI; Manning, IA) and were 20 days of age at the time of vaccination (Day 0). Both females and intact males were used. Animals were identified by uniquely numbered ear tags and acclimated with pen-mates prior to vaccination. Piglets were determined to be free from colonization by MHR as determined by MHR-specific PCR of nasal swabs collected on Day -6. Piglets were also determined to be free from antibodies to MHP and porcine reproductive and respiratory

syndrome virus (PRRSV) by ELISA (IDEXX Laboratories, Inc.; Westbrook, MA) from blood collected on Day -1. Pigs were given a prophylactic treatment of Excede® (Zoetis; Florham Park, NJ) six days prior to vaccination (Day -6) per label instructions. No other biologicals or pharmaceuticals were administered during the study other than vaccine and challenge. All animals were deemed to be healthy prior to vaccination. A diet of milk replacer was given until pigs were able to wean to non-medicated, dry food at which time water was provided *ad libitum*. Pigs were observed daily to ensure sufficient feed, water, and well-being.

The vaccination phase of the study was conducted at SLI. Pigs were initially housed in individual isolators until approximately two weeks of age at which time they were moved into brooders of two to three litter-mates each. Five days prior to challenge (Day 17), animals were transported to Veterinary Resources, Inc. (VRI; Cambridge, IA) for the challenge phase of the study. Pigs were again blocked by litter and housed for equal representation of treatment groups within pens where possible. During the challenge phase, NTX animals were housed separately from the challenged animals to prevent exposure to MHR. Pens were a raised deck with metal slatted flooring. Personnel involved in collecting data or performing laboratory assays were blinded to the allocation of pigs to treatment group.

Vaccine

Boehringer Ingelheim Vetmedica, Inc.'s (BIVI) MHR vaccine isolate originated from a pig with clinical signs of *Mycoplasma hyorhinis* infection. Initial isolation and speciation was performed in modified Friis media using standard techniques (10). For experimental vaccine production, McCoy cells (murine fibroblasts) in suspension were infected with MHR

and then subsequently inactivated with binary ethylenimine (BEI) per BIVI's proprietary outline of production.

A high, medium, and low dose vaccine were generated. The high dose vaccine contained the complete culture described above blended with Seppic Montanide™ ISA207 VG at 50% (w/w). For the medium and low dose vaccines, the MHR culture was first diluted 1:2 or 1:10, respectively, in phosphate buffered saline (PBS) before blending with adjuvant at 50% (w/w). The respective dose of MHR in the HIGH, MED, and LOW vaccine preparations was calculated to be 1.48×10^8, 7.41×10^7, and 1.48×10^7 CCU per 2.0 mL dose as determined by color changing units (CCU) from the pre-inactivation MHR harvest material.

A mycoplasma-free, adjuvanted placebo was generated which contained the exact composition as the high dose experimental vaccine, without MHR.

Pigs were administered a single 2.0 mL dose of vaccine or placebo intramuscularly (IM) in the right side of the neck on Day 0.

Challenge

A heterologous isolate of MHR was used for virulent challenge. The preparation of the infection dose has been previously described (see Chapter 2). The challenge dose was quantified by CCU.

Animals were administered MHR challenge as follows: 20 mL/intraperitoneal (IP) on Day 22, 10 mL/intravenous (IV) on Day 23, and 10 mL/intranasal (IN) on Day 24 for a total dose of 5.39×10^8 CCU per animal.

Observations

Animals were observed for general health daily prior to challenge. From four days prior to challenge (D18) through study termination (Day 43), all pigs were observed daily for clinical signs of MHR infection including respiratory distress, coughing, and lameness as described in Table 4.1. A pig was considered lame if it received a lameness score of ≥ 1 on any two (or more) consecutive days.

Table 4.1. Clinical observation scoring description.

Score	Respiration	Cough	Lameness
0	**Normal—**no respiratory discomfort	**Normal—**no cough	**Normal—**no visible lameness at a walk
1	**Mild—**mild increase in respiratory rate	**Mild—**slight cough that does not seem to disturb normal activities	**Mild—**difficult to observe lameness as the animal walks around the pen, not constantly lame when walking, walks at a normal speed, is weight bearing while walking and standing; lameness is indicated by intermittent reduced weight bearing on one limb or shortening of the stride.
2	**Moderate—**notable increase in respiratory rate	**Moderate—**loud, pronounced cough that disrupts normal activities	**Moderate—**constant and observed throughout every step at a walking pace, bearing some weight on the leg at a walk and standing, but short-striding one or more legs while walking, walks at a normal speed.
3	**Severe—**thumping	**Severe—**dry, hacking cough that appears painful	**Moderately Severe—**puts no weight on the leg the first few steps after standing, constant, obvious lameness while at a walking pace, putting very little weight on the leg at a walk or while standing; lameness requires the pig to slow its speed of walking.
4	N/A	N/A	**Severe—**will stand, may require assistance, for at least 3 minutes, non-weight bearing on one or more legs at walk or standing, still able to three-legged walk.
5	N/A	N/A	**Recumbent—**will not stand even with assistance.

Sample collection

Nasal swabs (eSwab™, Copan; Murrieta, CA) were collected on Day -6 (prior to antibiotic treatment and vaccination) and were tested for MHR by real-time PCR as previously described (see Chapter 2, pg. 25). Blood was collected on Day -1 (prior to vaccination) for MHP and PRRSV ELISA. Weights were collected on Day -1 (one day prior to vaccination), Day 21 (one day prior to challenge), and Day 42 (one day prior to necropsy). On Day 43, all pigs were anesthetized and euthanized via electrocution. A gross pathological evaluation was then performed. The carpi, tarsi, elbows and stifles from all animals were opened and examined for indications of mycoplasma-associated arthritis (e.g. excess or abnormal synovial fluid, abnormal synovial membranes, abnormal articular surfaces). A single, common swab (eSwab™, Copan; Murrieta, CA) was used to sample all articular and synovial surfaces of the carpi and tarsi for each pig. This procedure was repeated using a new swab for the four elbow and stifle joints for each pig.

The thoracic and abdominal cavities were examined for polyserositis (pleuritis, pericarditis, and peritonitis). Pericarditis was further scored on the following scale: **No pericarditis (0)**—smooth pericardial and epicardial membranes; **Mild (1)**—subtle roughening of the epicardium and noticeable thickening of the pericardial membrane, no adhesions present; **Moderate (2)**—obvious exudate and/or fluid in the pericardial sac and thickening of the epicardium and pericardium, adhesions present; **Severe (3)**—obliteration of the pericardial space due to exudates. After scoring, a swab (eSwab™, Copan; Murrieta, CA) of the pericardial surface (visceral) was collected from all pigs. Joint swabs and pericardial swabs were tested by MHR-specific real-time PCR.

Statistical analysis

All analyses were conducted by a BIVI statistician using SAS version 9.4 (SAS Institute Inc.; Cary, North Carolina). Analysis of abnormal respirations and coughing (ever present), lameness (two or more consecutive days post-challenge) and pericarditis (presence/absence) are reported (see Appendix C Table S24, S25, and S26, respectively). For each analysis, the preventive fraction (PF) and 95% confidence interval (CI) versus CTRL were estimated for each vaccine group. The GLIMMIX procedure of SAS was utilized to obtain parameter and variance/covariance estimates for the proportion affected for each treatment group. The model included the fixed effect of treatment group and random effect of pen. The analysis utilized a binomial distribution and logit link function. CI's were calculated using the delta method via the IML procedure of SAS. Weight measurements during the vaccination phase and challenge phase were analyzed separately. For the challenge phase, a repeated measures mixed model was utilized for the analysis of body weights using the MIXED procedure of SAS. The model included the fixed effect of treatment, day, and treatment by day interaction. Pen and litter within pen were included as random effects. An unstructured covariance structure was utilized. Least squares means (LSM) by treatment and day (see Appendix C Table 27) and the average daily gain (ADG) by treatment (see Appendix C Table 28), along with 95% CI's, are reported. Comparisons to the CTRL group are reported within day using alpha=0.05. Comparisons to the CTRL group for ADG are also reported using alpha=0.05. For the vaccination phase, data were analyzed using the MIXED procedure in SAS and the model included the fixed effect of treatment litter as a random effect. LSM by treatment, along with 95% CI's, are reported. Comparisons to the CTRL group are reported using alpha=0.05.

Results

Animals

Of the 120 pigs enrolled in this study, 110 of 112 animals in the vaccine/placebo groups and seven of eight NTX animals survived to the challenge phase of the study. One pig each in the NTX and LOW group were euthanized for humane reasons due to systemic bacterial infection (non-mycoplasma). A second pig in the LOW group died following complications with blood collection on Day 21.

Of the 117 pigs which survived to the challenge phase, three did not complete the study. Immediately following IV challenge on Day 23, a pig in the MED vaccine group exhibited anaphylaxis. Despite administration of epinephrine, the pig died shortly thereafter. A single pig in the placebo CTRL group was found dead eleven days post-challenge (Day 33). This pig had been observed with diarrhea on Days 31, 32, and 33. Upon necropsy, the elbow and stifle joints were noted with excessive fluid; focal areas of pleuritis, severe peritonitis and moderate pericarditis were observed. Diagnostic samples (including fresh and fixed colon, intestine, lung, and heart as well as a pericardial swab) were collected and sent to Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL) for further testing. A diagnosis of fibrinopurulent polyserositis was given with MHR as the causative agent. A pig in the HIGH vaccine group was found dead on Day 42. Upon necropsy, this pig exhibited pleuritis, peritonitis and severe pericarditis, as well as multiple joints with excess synovial fluid. Swabs from carpal, tarsal and stifle joints as well as a pericardial swab were collected and sent to ISU-VDL for further testing. Samples were positive for MHR and negative for *Haemophilus parasuis* by PCR. Death was attributed to virulent MHR challenge.

Clinical observations

Lameness was observed in a total of 30 animals. As seen in Figure 4.1, over half of the pigs were affected in the CTRL group with greater than 50% reductions in all vaccine groups. The HIGH and MED vaccine groups showed a significant reduction in lameness compared to the CTRL group with PF's of 0.64 (95% CI [0.10, 0.86]) and 0.76 (95% CI [0.26, 0.92]), respectively. The LOW group had a PF of 0.59 (95% CI [-0.01, 0.84]); however, with a lower CI < 0, this result was not significant. Lameness was noted as early as Day 24 and continued through study termination on Day 43. Two pigs in the CTRL group showed signs of lameness prior to challenge and were excluded from all analyses. As shown in Figure 4.2, the average duration of lameness for the CTRL group was 5.5 days while the HIGH, MED and LOW vaccine groups averaged 1.6, 1.0, and 2.7 days of lameness, respectively. Duration of lameness was considered a secondary measurement and therefore pairwise comparisons between groups were not performed.

Abnormal respirations were noted in twelve animals with at least one animal in each challenged group affected. These observations occurred as early as three days post-challenge (Day 25) and continued intermittently through to necropsy on Day 43. Only one pig (MED group) exhibited coughing post-challenge, and on only one day (Day 34). Abnormal respirations and coughing were combined for analysis and are shown in Figure 4.1. There were no significant differences between any vaccine group and the placebo CTRL group for abnormal respirations and coughing.

None of the NTX pigs exhibited any clinical signs in the post-challenge observation period.

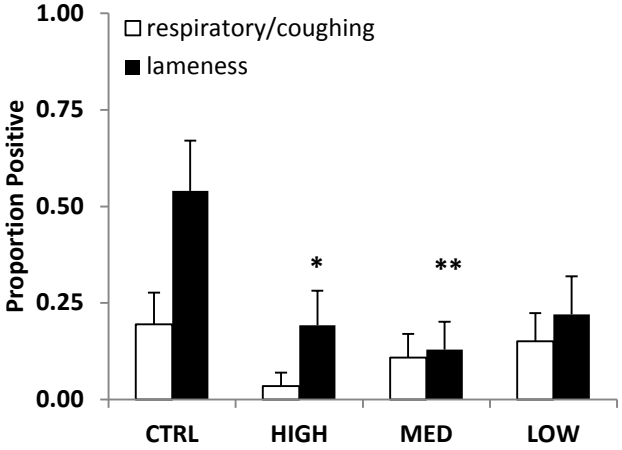

Fig.4.1. Clinical observations post-challenge. Observations of abnormal respirations and coughing were combined. A pig was deemed lame if it received a score of ≥ 1 on two or more consecutive days. CTRL = placebo control, HIGH = high dose vaccine, MED = medium dose vaccine, LOW = low dose vaccine; NTX group showed no clinical signs and are not represented. Data represents the least squares means for the proportion positive ± standard error. Significant reductions for lameness: *PF = 0.64 (95% CI [0.10, 0.86]), **PF = 0.76 (95% CI [0.26, 0.92]).

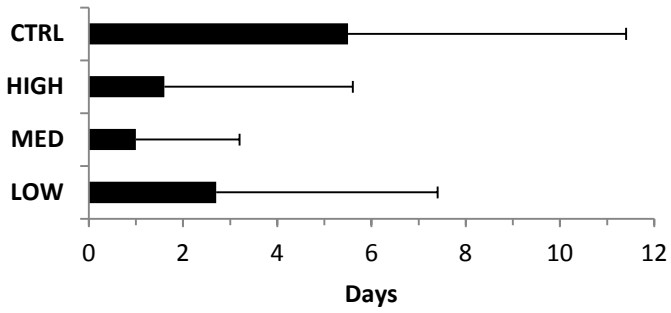

Fig. 4.2. Duration of lameness. Average number of post-challenge days with lameness score ≥ 1. CTRL = placebo control, HIGH = high dose vaccine, MED = medium dose vaccine, LOW = low dose vaccine; NTX group did not exhibit lameness and are not represented. Data represents the mean number of days with abnormal lameness score ± standard deviation.

Gross pathology

Pericarditis was observed in all challenged groups as shown in Figure 4.3. The CTRL group was most severely affected with all but one animal having pericarditis. All of the three vaccine groups showed a significant reduction of pericarditis with PF's of 0.52 (95% CI [0.26, 0.69]), 0.58 (95% CI [0.31, 0.74]), and 0.32 (95% CI [0.07, 0.50]) for the HIGH, MED, and LOW dose groups, respectively. Of all the animals with pericarditis, only one animal was noted with "mild" lesions while all remaining pigs fell into the "moderate" or "severe" categories.

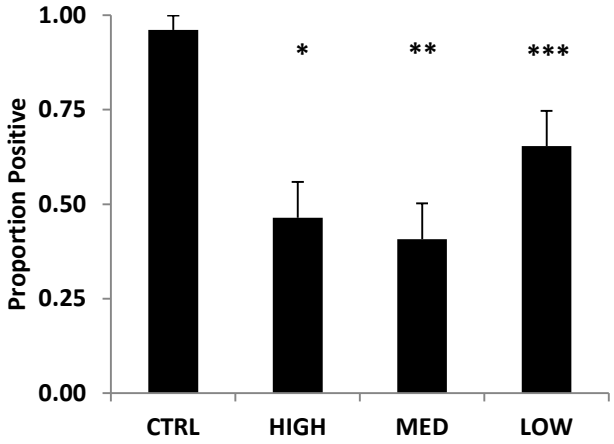

Fig. 4.3. Pericarditis. Pigs were observed for pericarditis at necropsy. CTRL = placebo control, HIGH = high dose vaccine, MED = medium dose vaccine, LOW = low dose vaccine; NTX group did not exhibit pericarditis at necropsy and are not represented. Data represents the least squares means for the proportion positive ± standard error. Significant reductions: *PF = 0.52 (95% CI [0.26, 0.69]), **PF = 0.58 (95% CI [0.31, 0.74]), ***PF = 0.32 (95% CI [0.07, 0.50]).

Pleuritis was present in 42% of the CTRL pigs and 7%, 30%, and 35% of the HIGH, MED and LOW dose vaccine group pigs, respectively. Peritonitis was observed in 54% of the CTRL pigs and 25%, 22%, and 19% of the HIGH, MED, and LOW dose vaccine group

pigs, respectively. The carpi, tarsi, elbows and stifles were examined for signs of arthritis. A pig was considered positive for arthritis if any joint was positive for arthritis. For the CTRL group, 96% of the pigs were affected; whereas for the HIGH, MED, and LOW dose vaccine groups, 21%, 19%, and 23% of the pigs were affected, respectively.

Pleuritis, peritonitis, and arthritis were considered secondary parameters and therefore pairwise comparisons between groups were not performed.

None of the NTX pigs exhibited polyserositis or arthritis.

Body weight

Figure 4.4A shows the average body weights for all challenged groups just prior to vaccination, just prior to challenge and just prior to necropsy. No significant differences were noted between vaccinates and placebo animals on Day -1 or Day 21. Following challenge, the HIGH, MED and LOW vaccine groups weighed an average of 7.83 lbs., 6.50 lbs., and 3.94 lbs. more than the CTRL group, respectively, on Day 42. The mean weights for HIGH, MED ($p < 0.0001$, each) and LOW ($p = 0.027$) vaccine groups were significantly greater than the placebo CTRL group at Day 42. NTX animals were not included in this analysis; however, measurements were collected and the mean weights and standard deviations were calculated to be 4.95 ± 0.50 lbs., 15.30 ± 2.97 lbs., and 42.21 ± 6.90 lbs. on Day -1, 21 and 42, respectively.

The average daily gain (ADG) was calculated during the challenge phase (Day 21 to 42) and is shown in Figure 4.4B. Similar to the Day 42 mean weight comparison, ADG's for the HIGH, MED ($p < 0.0001$, each) and LOW ($p = 0.0012$) vaccine groups were significantly higher than the placebo CTRL group.

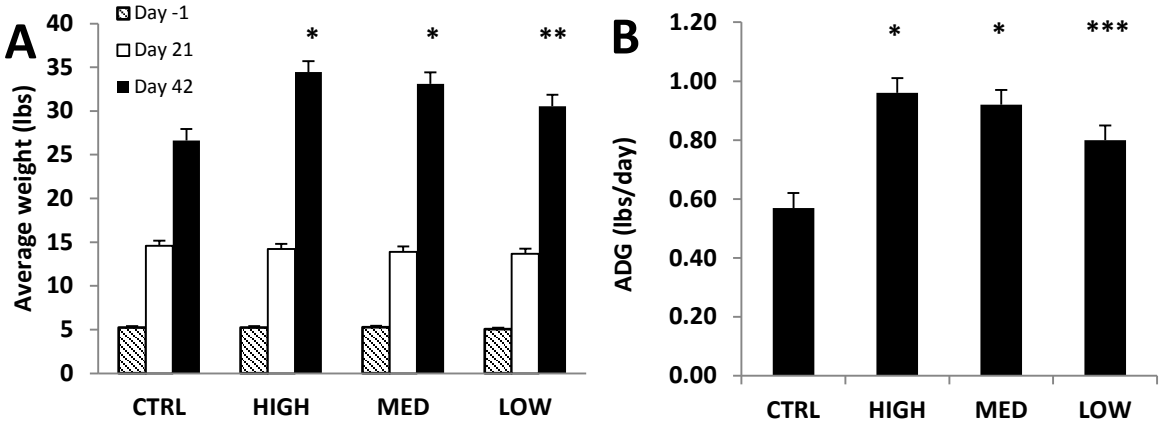

Fig. 4.4. Analysis of body weight. A. Mean weight one day prior to vaccination (Day -1), one day prior to challenge (Day 21), and one day prior to necropsy (Day 42). Data represents the least squares mean ± standard error. B. Average daily gain (ADG) for the challenge phase (Day 21 to 42). Data represents lbs/day ± standard error. CTRL—placebo control, HIGH—high dose vaccine, MED—medium dose vaccine, LOW—low dose vaccine; NTX group not included in analysis.* $p<0.0001$, ** $p=0.027$, *** $p=0.001$.

PCR

Pericardial and joint swabs were evaluated by MHR PCR. The CTRL group had the highest number of positive pericardial samples with 42% of the swabs testing positive for MHR. The number of positive pericardial swabs was fewer in the vaccine groups with 4% in the HIGH group and 15% in both the MED and LOW groups.

A pig was considered PCR positive for joint swabs if at least one of the two joint swabs collected was positive for MHR. The CTRL group had the highest number of positive pigs with 81% having one or more positive joint swabs. There were fewer numbers of positive animals in the vaccine groups with 29% of HIGH, 22% of MED, and 31% of LOW group pigs positive for one or more joint swab. PCR was considered supportive data and therefore pairwise comparisons between groups were not performed.

All pericardial and joint swabs from the NTX group were PCR negative for MHR.

Discussion

The objective of this study was to evaluate three different doses of BIVI's inactivated MHR vaccine for efficacy against virulent challenge in CDCD piglets at three weeks of age. All non-challenged NTX pigs remained free from clinical signs of MHR infection and all samples from this group were negative for MHR by PCR thus confirming biosecurity and a lack of exposure to environmental MHR. All but one pig in the placebo CTRL group had pericarditis and over half of the pigs in this group were lame, indicating an effective MHR challenge.

The HIGH, MED and LOW doses of the vaccine all showed significant reductions of pericarditis compared to the CTRL group. For lameness, only the HIGH and MED doses of vaccine had positive PF values with lower CI's > 0, showing significant reductions compared to the CTRL group. When looking at the results for clinical observations (Fig. 4.1) and pericarditis (Fig. 4.3), a titration effect of the vaccine is apparent. In some instances such as lameness and pericarditis, the MED dose vaccine slightly out-performed the HIGH dose vaccine; whereas, in the case of weight gain, the reverse is true. In all instances, the HIGH and MED doses of vaccine performed better than the LOW dose vaccine. Still, the LOW dose vaccine was efficacious and did provide a significant reduction of pericarditis and significant increase in weight gain.

Manifestations of respiratory symptoms were minimal (Fig. 4.1.) despite the fact that IN inoculation was included as part of the challenge protocol. Previous reports indicate that MHR can be frequently isolated from pneumonic lungs (5, 11, 12); however, our respiratory findings are in agreement with the clinical signs and lesions described by Rovira *et al* (2010).

It should be noted we have since refined our challenge model to omit the third day IN challenge (see Chapter 2).

The incidence of polyserositis was higher in this study than observed in our previous experiments (see Chapter 2 and 3). The severity and frequency of pericarditis (25 of 26 CTRL pigs affected) supports a highly virulent challenge. It is possible that the age of the animals played a role in this observed increase. A large portion of our previous challenge model work was evaluated in seven-week old pigs; whereas, in this study pigs were six-weeks of age at challenge. We have already demonstrated susceptibility to MHR-associated pericarditis is greatly reduced after seven weeks of age (see Chapter 3). Perhaps pigs younger than seven-weeks of age are even more at risk.

The MHR PCR results are similar to past findings (see Chapter 2 and 3) where a lower number of MHR PCR positive results are obtained from pericardial swabs than joint swabs. We attribute this phenomenon to the progression of disease for MHR. Although the pathogenesis of MHR is as yet poorly defined, our results suggest that systemic infection initially and drastically affects the heart. The bacteria then disseminate to the rest of the body (liver, brain, kidneys, etc.) including a prolonged and chronic infection of the joints. Our post-challenge phase is three weeks in length to allow for sufficient time for lameness to manifest, and we propose that MHR is in the process of being cleared from the pericardial cavity by the time of necropsy. Direct pairwise comparisons of the number of MHR PCR positive samples were not performed, but vaccinated animals did show reductions of MHR PCR positive samples compared to the placebo group for both pericardial swabs (vaccinates $\leq$ 15 %, CTRL = 42%) and joint swabs (vaccinates $\leq$ 31 %, CTRL = 81 %). It may be

possible that our vaccine reduces the numbers of MHR which reach the target tissues; however, our PCR is qualitative and total bacterial load was not measured. Quantitative assays have since been developed (2) and may be utilized in future studies to evaluate such parameters.

To conclude, this study has proven our inactivated *Mycoplasma hyorhinis* vaccine administered to three-week old CDCD piglets to be efficacious. We demonstrated a minimum immunizing dose of MHR (7.41×10^7 CCU per 2.0 mL dose) provided significant reductions of both lameness and pericarditis and a significant increase in weight gain compared to non-vaccinated pigs.

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B.M., W.Z., and K.B. were involved with study design and management. R.C. and J.K. were involved with study design and approvals. L.B. performed statistical analyses. F.C.M. edited the manuscript. B.M. managed laboratory testing and review. B.M. wrote the manuscript which has been read and approved by all authors.

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CHAPTER 5. *MYCOPLASMA HYORHINIS* PROTEOMIC ANALYSIS: DIFFERENTIAL EXPRESSION BETWEEN IN VITRO AND EX VIVO CULTURES

A paper prepared for submission to a peered reviewed journal

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Abstract

Background

Mycoplasma hyorhinis (MHR) is a pathogen of swine causing polyserositis and arthritis. It is also a common contaminant of tissue culture in the laboratory setting. We sought to exploit the later by evaluating protein expression of a single isolate of MHR cultured in two *ex vivo* systems and comparing to a standard broth culture.

Results

Of the 658 predicted proteins in the MHR reference proteome HUB-1, 291 were identified in this study. The average protein coverage was 30% with a false discovery rate of ≤ 1.3%. We confirmed the existence of 35 hypothetical proteins and identified several putative membrane-associated and transporter proteins which were up-regulated in the two *ex vivo* cultures compared to broth culture of the same isolate.

Conclusion

Our results demonstrate differential expression of particular MHR proteins in two *ex vivo* systems. Proteins up-regulated in tissue culture may provide insight into the mechanisms necessary for *in vivo* infection and are potential candidates for vaccine targets.

Background

Mycoplasma hyorhinis (MHR) is a pathogen of swine causing polyserositis, lameness and arthritis in young pigs (1-3). As no commercial vaccines are currently available, treatment consists of antibiotic therapy. The pathogenesis and virulence factors of MHR are not well understood, making it difficult to identify potential vaccine targets. We have defined an *in vivo* model to establish virulence of MHR isolates (see Chapter 2), thus allowing for evaluation of vaccine candidates.

MHR is a common contaminant of tissue culture and some strains can be adapted to growth on certain cell lineages (4-9). In this study we sought to evaluate the proteome of two *ex vivo* cultures of a single isolate of MHR for differential expression as compared to standard broth culture. Madin-Darby canine kidney (MDCK) adherent epithelial cells and McCoy mouse fibroblast cells in suspension (non-adherent) were utilized for the tissue culture systems. A modified Friis media (10) was used for the broth culture. One dimensional liquid chromatography nanospray ionization tandem mass spectrometry (1D-LC NSI MS/MS) was utilized to evaluate the cultures in a manner similar to a previous study performed with *Mycoplasma hyopneumoniae* (11). The genome of *M. hyorhinis* HUB-1 was sequenced by Liu *et al* in 2010 (12), has 658 protein-encoding genes, and was used as a reference for our cultures. Results from this study will help to further our understanding of the mechanisms MHR employ to cause infection in biological systems.

Results

Identified proteins

Protein coverage averaged 23.4%, with 29.3 mass spectrum matches and 6.5 unique peptide sequences per protein. False discovery rates ranged from 1% to 1.3% across all samples. Overall, 291 of 658 known and predicted MHR HUB-1 proteins were identified, representing 44% of the proteome. Tables S34, S35, and S36 contain the proteins identified in each culture type, along with coverage calculations and the number of unique spectra and peptides for each protein. Thirty-five proteins designated as hypothetical were identified in this analysis, confirming their existence. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (13) with the data set identifier PXD004533 and 10.6019/PXD004533. Included in the submission are detailed peptide identifications from this study using the filter criteria presented in the methods section.

Isolating MHR from host cells and obtaining proteome coverage comparable to broth grown cells presented a challenge due to host cell contamination. Similar proteome coverage is essential in performing a valid differential expression analysis. Table 5.1 shows the number of MHR proteins identified in the broth, MDCK, and McCoy cultures, along with the average coverage, unique spectra, and unique peptides per protein. Histograms (Figure 5.1) were generated to further illustrate the consistency of the proteomics for the three culture types. These histograms largely overlap, indicating that a similar depth of proteome coverage was achieved for each.

Table 5.1. Protein identification metrics. Total number of proteins. Averages for protein coverage, number of unique spectra, and number of unique peptides.

Culture Type	Number of Proteins	Average % Coverage	Average Number of Unique Spectra	Average Number of Unique Peptides
Broth	234	23.2	32.5	6.6
MDCK	252	23.8	34.9	6.6
McCoy	201	23.2	20.4	6.3
Combined	229	23.4	29.3	6.5

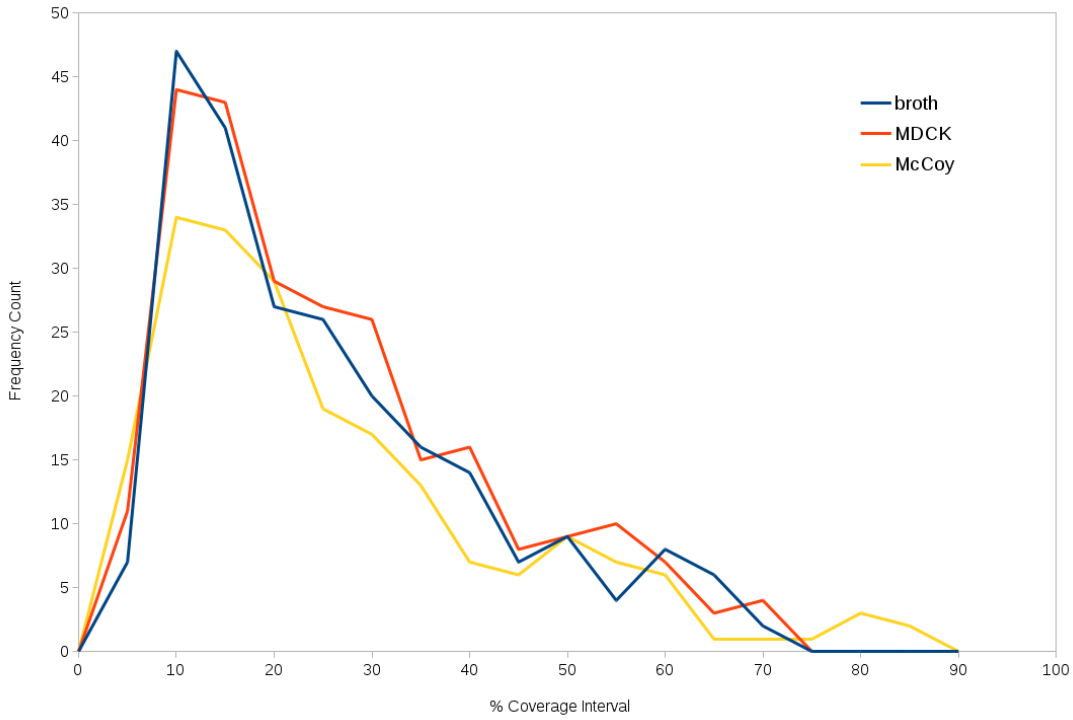

Figure 5.1. Histograms of protein coverage. Broth = modified Friis media; MDCK = Madin-Darby canine kidney adherent epithelial cells; McCoy = mouse fibroblast cells in suspension (non-adherent).

Differential expression

Comparisons of the identified proteins were made between broth vs. MDCK cultures, broth vs. McCoy cultures, and MDCK vs. McCoy cultures. For the broth vs. McCoy comparison, 99 proteins showed a significant difference ($p < 0.05$) in expression. For the broth vs. MDCK comparison, 76 proteins showed a significant difference ($p < 0.05$) in expression. The comparison for MDCK vs. McCoy identified 76 proteins with a significant difference ($p < 0.05$) in expression levels.

Figure 5.2 is a Venn diagram which illustrates the overlap of the proteins identified as significant ($p < 0.05$) for differential expression in each of the three comparisons. Appendix D: Table S29 lists the 34 proteins uniquely identified to the broth vs. McCoy cell comparison. Of these proteins, eight were up-regulated and 26 were down-regulated in McCoy cultures. All up-regulated proteins showed a ≥ 2.7 fold increase, with six of the eight proteins at a ≥ 5.7 fold increase in expression (note that all fold changes are expressed as log2). The 26 down-regulated proteins varied from a 1.2 to 10.5 fold decrease in expression in McCoy cells, with sixteen proteins showing a ≥ 6.4 fold decrease in expression.

Appendix D: Table S30 lists the 20 proteins uniquely identified to the broth vs. MDCK comparison. Of these proteins, 16 were up-regulated and four down-regulated in MDCK cultures. The up-regulated proteins ranged from a 0.8 to 9.9 fold increase, with six proteins showing a ≥ 8.5 fold increase. The down-regulated proteins ranged from a 0.9 to 9.9 fold decrease in expression in MDCK cells.

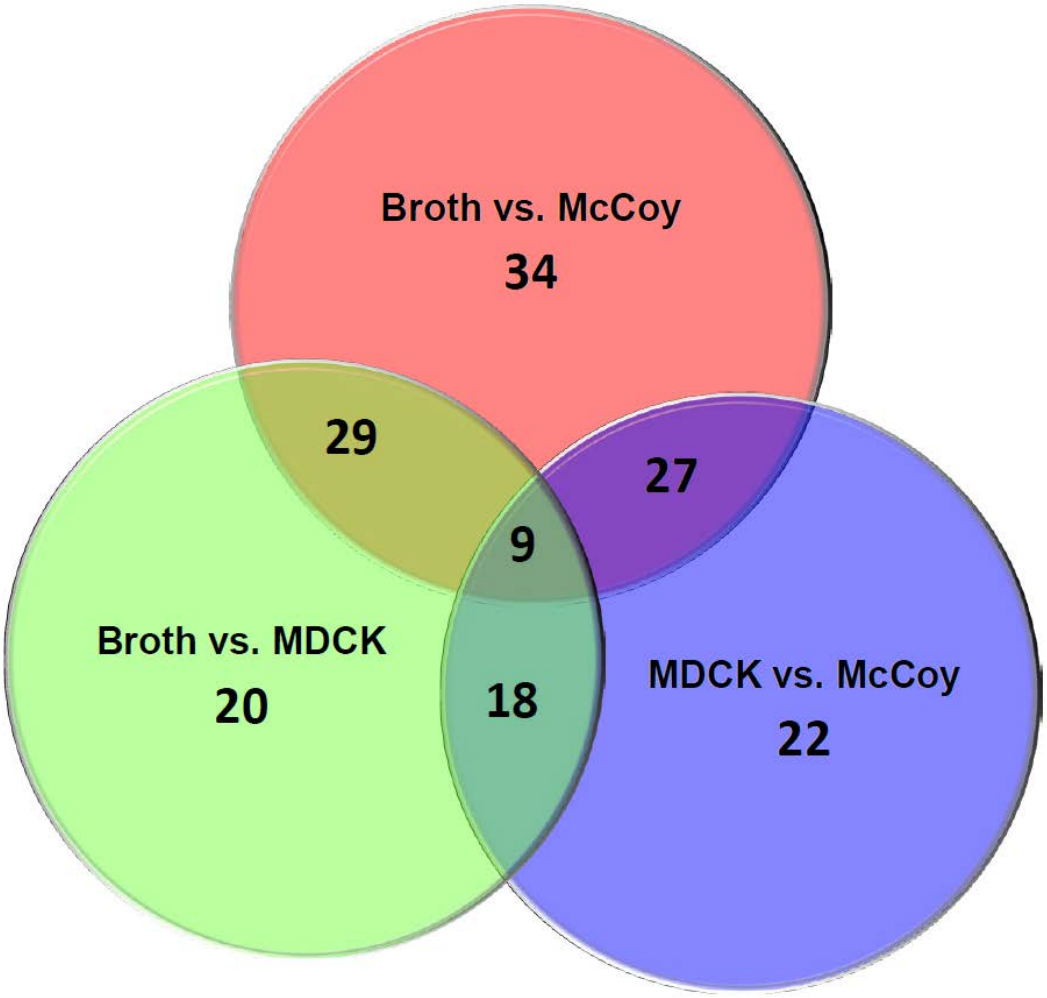

Figure 5.2. Venn diagram for intersection of comparisons for differential expression. Broth = modified Friis media; MDCK = Madin-Darby canine kidney adherent epithelial cells; McCoy = mouse fibroblast cells in suspension (non-adherent). Comparisons were made using the first term as the control and the second term as the experimental treatment with the numbers representing the number of proteins significant ($p < 0.05$) for differential expression (over or under) in the treatment group.

Appendix D: Table S31 lists the 22 proteins uniquely identified to the MDCK cell vs. McCoy cell comparison. Only two of these proteins were up-regulated, showing a 2.5 and a 7.4 fold increase in expression in McCoy cells. The 20 down-regulated proteins ranged from a 0.6 to 9.2 fold decrease in McCoy cells, with 5 proteins showing a $\geq$ 5.6 fold decrease in expression.

The center of the Venn diagram (Fig. 5.2) lists nine proteins common to all three comparisons (see Appendix D: Table S32). Twenty-nine proteins were identified in the overlap of the comparisons of broth culture to each of the *ex vivo* systems and all showed identical direction of expression (over/under) in both MDCK and McCoy cell cultures as listed in Appendix D: Table S33. Six of the 29 proteins were up-regulated in both cell types with similar levels of expression, ranging from a 2.1 to an 8.5 fold increase in McCoy cells and a 1.8 to a 9.2 fold increase in MDCK cells. The 23 down-regulated proteins also showed similar levels of expression, ranging from a 1.3 to 10.4 fold decrease in McCoy cultures; whereas, under expression in MDCK cells ranged from a 0.8 to 10.8 fold decrease.

Discussion

Our hypothesis was that *ex vivo* cultures more closely resemble *in vivo* infection by MHR and that potential vaccine targets could be identified in this study for further evaluation in the host animal. As such, most of our interests lie in the up-regulated proteins from the *ex vivo* cultures. However, the down-regulated proteins also help to expand the understanding of MHR biology.

As shown in Figure 5.1 and Table 5.1, proteome coverage was similar for each culture type. One of the challenges with multiple species proteomics is contamination leading to decreased proteome coverage of one or more species involved. Based on quality and consistency of the proteomics across all three culture types, the isolation of MHR from MDCK and McCoy cells was very effective. Broth and MDCK cultures were similar in all aspects listed in Table 5.1 (number of proteins, protein coverage, unique spectra and peptide). McCoy cultures resulted in a 40 % reduction in the average number of spectra per protein, 17 % fewer proteins, but only a 5 %

reduction in peptides per protein and no difference in protein coverage. The reduction in number of spectra per protein likely results from competition with non-MHR proteins for mass spectrometry analysis time. It could also be that fewer MHR proteins are expressed when McCoy cells are used as a host. Figure 5.3 is a heat map of differentially expressed proteins only. Hierarchical clustering of replicates indicates good reproducibility within each culture type.

Among the up-regulated proteins in the comparisons are several predicted transporter and membrane-associated proteins. These proteins would likely be surface exposed with antigenic regions accessible by host antibodies making them viable candidates for vaccine evaluations.

For the broth vs. McCoy comparison (Appendix D: Table S29), an ABC transporter permease (gene MHR_0064), a hypothetical protein (gene MHR_0453) with predicted hydrolase activity, and a hypothetical protein (gene MHR_0655) with predicted transmembrane regions (14) all showed ≥ 8.4 log increase in expression. The cargo for the 74 kDa transporter permease is unknown, as is the substrate for the 33 kDa predicted hydrolase. The P59-like protein encoded by gene MHR_0073 showed a 5.7 fold increase in expression in McCoy cell cultures. This 57 kDa protein contains two ABC transporter domains (14); however, this transport function is currently hypothetical and the potential cargo unknown. There were five hypothetical proteins down-regulated in McCoy cell cultures: three with predicted transmembrane regions (genes MHR_0628, MHR_0285, and MHR_0630), one (gene MHR_0293) with putative hydrolase activity, and the last (gene MHR_0508) a probable transcriptional regulator (14). All five of these proteins were down-regulated from 2.0 to 9.2 fold. The probable transcriptional regulator is predicted to have a cytoplasmic locale (14). Transcriptional regulation in MHR has not been thoroughly evaluated (15). It is unclear whether this probable regulator induces or represses transcription, but given that the proteins encoded by *dnaX* (MHR_0101) were up-regulated 7.8

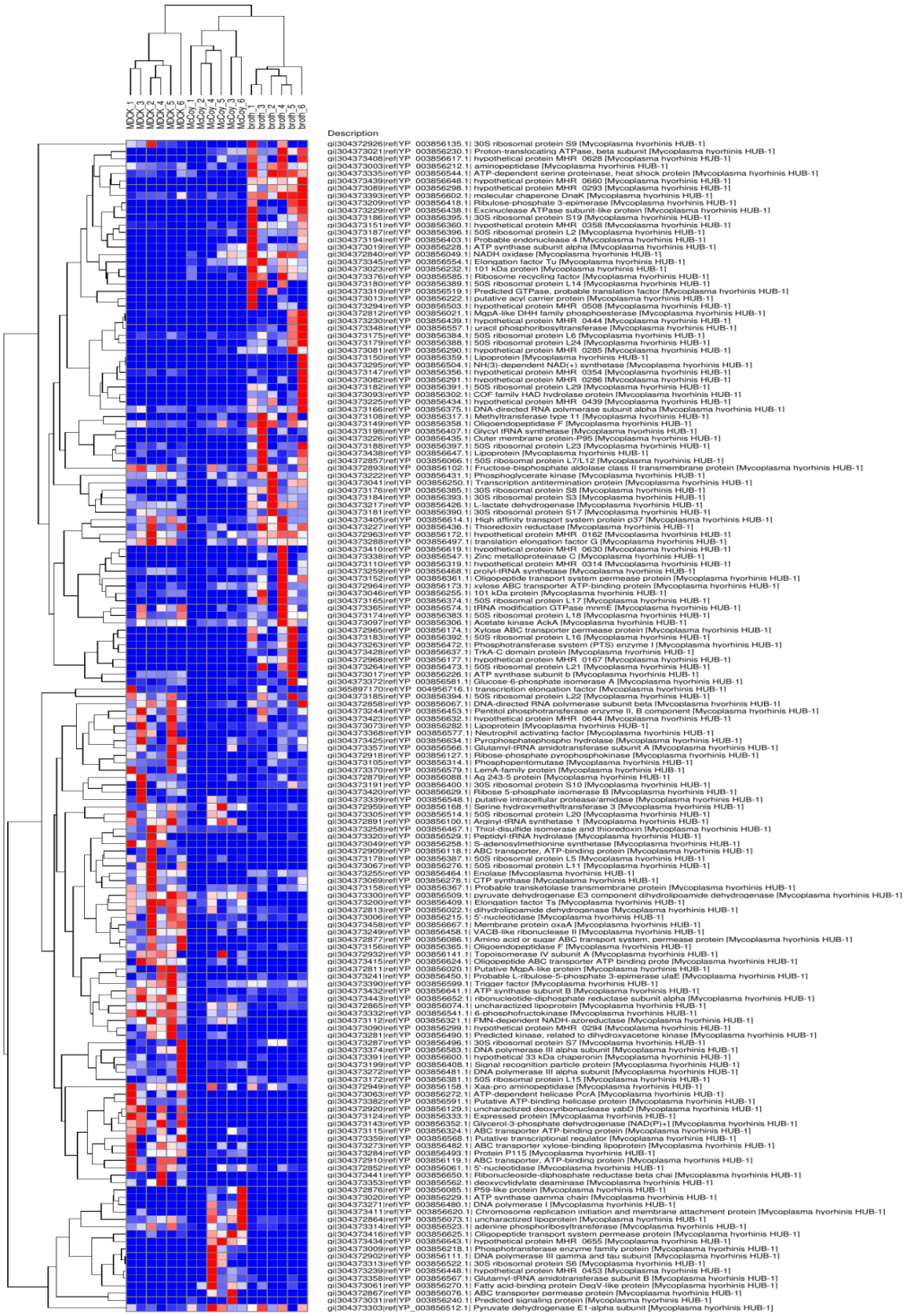

Figure 5.3. Heat map for distribution of proteins among the culture types which were significant ($p<0.05$) for differential expression. Red indicates up-regulated and blue indicates down-regulated.

fold, DNA syntheses was likely occurring, offering numerous potential binding sites. If it is a repressor, it's reduced expression would lead to up-regulation of the target gene and could point to regulation of any one of the eight up-regulated proteins, including DNA polymerase III. Regardless, the signal to turn this putative regulator on or off is unknown. The Trka-C domain protein encoded by *trkA* (MHR_0649), a predicted transmembrane transporter for potassium ions (14), and the oligopeptide transport permease encoded by *oppF* (MHR_0359) were each down-regulated in McCoy cultures 8.3 fold and 8.9 fold, respectively. It is difficult to interpret the reduced need for these two transporters in the McCoy cultures as this may simply be due to the levels of potassium and availability of amino acids in the culture medium. Lastly, the lipoprotein encoded by MHR_0357 was down-regulated 10.5 fold. Such a large drop in expression in McCoy cell cultures may indicate that this lipoprotein does not play a vital role in the infection process, possibly acting as a decoy antigen in the host as has been shown for some of the variable lipoproteins (Vlp's) for MHR (16).

The broth vs. MDCK comparison (Appendix D: Table S30) identified a glycerol-3-phosphate dehydrogenase encoded by *gpsA* (MHR_0350), which was up-regulated 9.9 fold in MDCK cultures. This enzyme plays a major role in carbohydrate metabolism and lipid biosynthesis (17, 18) and has been shown to be associated with the inner cytoplasmic membrane for *E. coli* (18). The protein encoded by gene MHR_0074 is a putative amino acid or sugar ABC transporter with several predicted transmembrane regions (14). This 66 kDa permease had a 9.1 fold increase in expression in MDCK cultures. A LemA-family protein (gene MHR_0589) and oligoendopeptidase F (gene MHR_0363, *pepF*) were also up-regulated, 2.9 and 2.6 fold, respectively. The LemA protein has predicted transmembrane regions with homology to a two-component regulatory system in a single protein (14, 19). Hrabak and Willis (1992)

demonstrated the requirement of this protein for *Pseudomaonas syringae* pv. syringae to cause disease in bean plants. The function of LemA in MHR has not been evaluated. Oligoendopeptidase F is a metalloendopeptidase requiring metal ions for the hydrolysis of peptide bonds (20). An ortholog of this protease has been identified in *M. hyopneumoniae* and has been found to cleave bradykinin which may assist with survival in the host (21). The role of this protein has not been investigated in MHR. The down-regulated proteins in the MDCK cultures include a 96 kDa lipoprotein (gene MHR_0659) and a xylose ABC transporter permease (*xylH*) which were down-regulated 2.7 and 9.9 fold, respectively.

There were only two up-regulated proteins identified in the MDCK vs. McCoy comparison (Appendix D: Table S31). One of these was a ribosomal protein. The other was an oligopeptide transport permease encoded by *oppC* and up-regulated 2.5 fold in McCoy cultures compared to MDCK cultures. This ABC transporter has several predicted transmembrane regions (14). Conversely, the oligopeptide transporter encoded by *oppD* showed a 2.1 fold decrease in McCoy cultures compared to MDCK cultures. Several transmembrane proteins and transporters, as well as one hypothetical protein with predicted transmembrane regions, were also down-regulated in McCoy cell cultures compared to MDCK cell cultures. It is unknown why these proteins might be unnecessary, or less necessary, in McCoy cultures as compared to MDCK cultures. While both represent mammalian cells, differences between the tissue culturing protocols may be possible explanations. The growth medium was modified essential media (MEM) for the MDCK cells and Dulbecco's modified essential media (DMEM) for the McCoy cells. Both were supplemented with fetal bovine sera. Although all of the components of MEM are present, DMEM is enriched with additional amino acids (including asparagine, aspartic acid, glutamic acid, proline and serine), vitamins, and inorganic salts (www.sigmaaldrich.com). It is

also likely that the difference between anchored cells versus those in suspension require distinctive means for infection by the mycoplasmas. Cell type (epithelial vs. fibroblast) and species host (canine vs. murine) may also influence outcomes.

There were 29 proteins which overlapped the two broth vs. *ex vivo* comparisons (Appendix D: Table S33). These proteins showed identical direction of regulation (up/down) in both MDCK and McCoy cultures. Six of these proteins were up-regulated in both *ex vivo* cultures, with arginyl-tRNA synthetase showing the greatest fold increase in both cell types compared to broth. Although MHR does not utilize arginine for an energy source, the increased expression for this particular tRNA ligase would indicate a greater need by MHR for arginine in *ex vivo* systems. Adenine phosphoribosyltransferase (*apt*) was similarly up-regulated in both systems at a 2.8 to 3.3 fold increase. This enzyme plays a role in the salvage pathway, important in bacteria such as MHR which lack *de novo* synthesis pathways for nucleic acid precursors. An uncharacterized lipoprotein of 88 kDa (gene MHR-0061) showed similar over expression in MDCK cultures (2.0 fold increase) and McCoy cultures (2.6 fold increase). The function of this lipoprotein is not yet defined. Contrary to the enzyme identified in the broth vs. MDCK comparison, a second oligoendopeptidase F (gene MHR_0356, *pepF*) was identified as being slightly down-regulated (1.3 to 1.8 fold) in both tissue culture systems. Additionally, a zinc metalloproteinase C (gene MHR_0553) was down-regulated in both systems but to a greater extent (8.6 to 8.9 fold) than the second *pepF* protein. The DnaK heat-shock chaperone protein was mildly down-regulated in both cell cultures (1.7 to 2.4 fold). Seven hypothetical proteins were also down-regulated in both *ex vivo* cultures, varying from 3.3 fold to 10.8 fold under expression. Lastly, the outer membrane protein P95 (gene MHR_0440) was down-regulated 8.5 fold in MDCK cultures and 8.1 fold in McCoy cultures. This protein has been identified as an

ortholog to the *M. hyopneumoniae* P97-like adhesin (22). The *M. hyopneumoniae* P97 protein has been shown to bind to ciliated epithelial cells (23). As a functional ortholog, it may be likely that P95 is not needed for attachment to the non-ciliated MDCK and McCoy cells.

Besides the proteins identified above, previous MHR research offers additional proteins of interest. The MHR lipoprotein P37 has been thoroughly investigated with implications in human carcinoma and cancer invasiveness (24-28). In our study, this high affinity transport system protein encoded by gene MHR_0625 was identified in the Broth vs. McCoy and MDCK vs. McCoy comparisons and falls into the 27-protein overlap in the Venn diagram (Fig. 1). The P37 protein was significantly ($p < 0.05$) down-regulated 2.8 fold in the broth vs. McCoy comparison and 2.7 fold in the MDCK vs. McCoy comparison. Given the plethora of research for P37, perhaps this protein has a tropism for cancer cells and simply was unnecessary for infection of murine fibroblasts.

Mycoplasma hyorhinis possess a set of variable lipoproteins (Vlp's) which undergo antigenic variation, may be important for host evasion, and have been shown to vary in repertoire number by isolate (12, 16, 29-33). Our reference strain, HUB-1, possesses all seven known *vlp* genes in the order 5'-*vlpD*-*vlpE*-*vlpF*-*-IS-*-*vlpG*-*vlpA*-*-IS-*-*vlpB*-*vlpC*-3' (12, 34) where 'IS' are insertion sequences and '*' are genes for uncharacterized or hypothetical proteins (in 5' to 3' order: MHR_0340, MHR_0342, MHR_0345, and MHR_0347). The MHR isolate used in this study has not been fully characterized to determine which *vlp* genes are present. No Vlp's were noted in our analyses, likely an artefact of our methodology. It is not possible to determine from our data whether Vlp expression changes in these two specific tissue culture systems. Transcriptional profiling may assist in identifying the presence or absence of mRNA in future studies.

Conclusions

To the best of our knowledge, this is the only differential proteomic analysis comparing *in vitro* and *ex vivo* cultures of *Mycoplasma hyorhinis*. Thirty-five hypothetical *M. hyorhinis* proteins have been confirmed. We identified several putative membrane-associated and transporter proteins which were up-regulated in two *ex vivo* cultures compared to a broth culture of the same isolate. These proteins provide candidates for further investigation in future *in vitro* and *in vivo* studies.

Materials and Methods

Sample preparation

The *M. hyorhinis* (MHR) isolate used in this study originated from the joint fluid of a clinically affected pig. Isolation occurred in a modified Friis media (10) supplemented with 10 % (v/v) porcine serum (Life Technologies, Grand Island, NY) and 5% (v/v) yeast extract (Life Technologies, Grand Island, NY). Purity was confirmed by aerobic and anaerobic culture on sheep blood agar and identity was confirmed by real-time PCR, amplifying the 16S rRNA gene. A low passage (pass 7) stock was titrated by color changing units (CCU) assay and had a resulting titer of 7.63 $\log_{10}$ CCU/mL. This stock was used to inoculate the broth and tissue cultures for LC-MS/MS analysis.

For broth cultures, six 50 mL conical tubes (BD Biosciences, Bedford, MA) each containing 28.0 mL of modified Friis media were inoculated with 2.0 mL per tube of MHR. Caps were sealed tightly and all six tubes were incubated at 37 °C while shaking at 100 rpm. Cultures were allowed to incubate for five days (~120 hours) to reach peak CCU at late log phase, as

indicated by pH shift (acidification) of the growth media. All six samples were harvested by freezing at < -60 °C without cryo-preservative.

Two sets of *ex vivo* cultures were produced, one composed of adherent monolayers of Madin-Darby canine kidney (MDCK) cells and one as a suspensions of McCoy cells (murine fibroblasts). For MDCK cultures, cells were inoculated in six 75 cm^2 vented culture flasks (BD Biosciences, Bedford, MA) in minimum essential media (MEM; Life Technologies, Grand Island, NY) supplemented with 5 % FBS (fetal bovine serum; SAFC, Lenexa, KS). Flasks were incubated at 37 °C + 5 % CO_2 to ≥ 98 % confluency. Prior to infection, media was removed and cells were rinsed with 0.1 M phosphate buffered saline (PBS; Life Technologies, Grand Island, NY). Mycoplasmas were then added at 2.0 mL per flask and allowed to incubate at 37 °C for at least two hours prior to adding 28.0 mL per flask of pre-warmed media consisting of MEM supplemented with 2 % FBS. Cultures were allowed to incubate as previous for five days (~120 hours) to reach peak CCU, as indicated by ≥ 90 % cytopathic effect (CPE) of the cell monolayers. All six samples were harvested by freezing the flasks at ≤ -60 °C for two hours and thawing in a 37 °C incubator. Cell lysate from each flask was then transferred to individual 50 mL conical tubes and frozen at ≤ -60 °C without cryo-preservative.

For McCoy cultures, cells were inoculated into six 125 mL disposable spinner flasks (Corning Inc. Life Sciences, Lowell, MA) in Dulbeco's minimum essential media (DMEM; SAFC, Lenexa, KS) supplemented with 5 % FBS and 2.5 % HEPES (Life Technologies, Grand Island, NY) for a total volume of 28.0 mL per flask. Cells were allowed to incubate for three days at 37 °C while stirring at 100 rpm before inoculating each flask with 2.0 mL of MHR directly into the cell suspension. Cultures were allowed to incubate as previous for four days (~96 hours) to reach peak CCU, as indicated by a drop in McCoy cell viability to below 80 % as

measured by a Vi-CELL® cell analyser (Beckman Coulter, Jersey City, NJ), indicating the presence of CPE. For harvest, the contents of the six flasks were transferred to individual 50 mL conical tubes and frozen at ≤-60 °C without cryo-preservative.

Representative samples of each culture type were confirmed to be MHR by real-time PCR and by western blotting using MHR specific, anti-P70 monoclonal antibody purchased from Dr. Kim Wise, University of Missouri (35). Titers of each culture type at the time of freezing were 7.6 $\log_{10}$ CCU/mL, 6.8 $\log_{10}$ CCU/mL, and 8.5 $\log_{10}$ CCU/mL for the broth, MDCK and McCoy cultures, respectively. Protein concentrations were 6.11 mg/mL, 3.45 mg/mL, and 3.07 mg/mL for broth, MDCK and McCoy cultures, respectively, as measured using Quant-IT protein assay kit and Qubit fluorometer (Life Technologies, Grand Island, NY). Representative samples of Mycoplasma-free control cultures were MHR PCR and P70 negative.

MHR culture preparations involved no vertebrates for this study and required no ethical committee approvals. The research was performed according to the guidelines and standards of Boehringer Ingelheim Vetmedica, Inc. (BIVI), the University of Arizona, and Iowa State University.

Mass spectrometry

Infected MDCK and McCoy cultures were gently rinsed with PBS and centrifuged at 3000 RCF, 4 ºC for 5 minutes. The supernatant was removed and an additional 5 ml of PBS was added. Cultures were vortexed vigorously to suspend free MHR cells and then centrifuged at 3000 RCF, 4 ºC for 5 minutes. The supernatant containing suspended MHR cells was transferred to high speed centrifuge tubes and centrifuged at 20,000 RCF, 4 ºC for 1 hour. Pelleted cells were rinsed with PBS and centrifuged at high speed once again to re-pellet. The supernatant was

removed and pelleted MHR cells were resuspended in 500 µl hexafluoroisopropanol (HFIP) for cell lysis. Tubes were bath sonicated for 30 seconds and vortexed to completely resuspend and lyse the cells. The lysate was transferred to 2 ml screw top tubes for further processing.

Broth cultured MHR were centrifuged at 20,000 RCF, 4 ºC for 1 hour and the supernatant discarded. Cells were washed using 5 ml PBS, centrifuged again and the supernatant discarded. Pelleted cells were resuspended in 500 µl HFIP. Tubes were vortexed to completely resuspend and lyse the cells and the lysate was then transferred to 2 ml screw top tubes for further processing.

HFIP was subsequently removed using vacuum centrifugation. Dried cell lysate was rehydrated with the addition of 5 µl of 100 mM ammonium bicarbonate (ABC). Five µl of 100 mM dithiothreitol in 100 mM ABC was added and samples were placed at 85 ºC for 5 minutes to reduce disulfide bonds. Once returned to room temperature, 5 µl of 100 mM iodoacetamide was added to alkylate the reduced disulfide bonds and samples were placed in the dark for 30 minutes. Two µg proteomics grade trypsin (T6567, Sigma, St. Louis, MO) was added to each sample and the total volume was raised to 100 µl using 100 mM ABC. Samples were incubated at 37 ºC overnight. Following digestion, samples were desalted using a reverse phase peptide trap (TR1/25108/52, Michrom Bioresources, Auburn, CA) according to the manufacturer's instructions. Purified peptides were dried using vacuum centrifugation, resuspended in 3 µl of 2 % acetonitile (ACN), 0.1 % formic acid (FA) in water and transferred to HPLC vials for further analysis.

Separation of peptides was performed using a Dionex U3000 splitless nanoflow HPLC system operated at 333 nl minute using a gradient from 2 –50 % acetonitrile over 60 minutes,

followed by a 15 minute wash with 95 % acetonitrile and a 15 minute equilibration with 2 % acetonitrile. The C18 column, an in-house prepared 75 μm × 15 cm reverse phase column packed with Halo 2.7 μm, 90 Å C18 material (MAC-MOD Analytical, Chadds Ford, PA) was located in the ion source just before a silica emitter. A potential of 2100 volts was applied using a liquid junction between the column and emitter. A Thermo LTQ Velos Pro mass spectrometer using a nanospray Flex ion source was used to analyze the eluate from the U3000. Scan parameters for the LTQ Velos Pro were one MS scan followed by 10 MS/MS scans of the 5 most intense peaks. MS/MS scans were performed in pairs, a CID fragmentation scan followed a HCD fragmentation scan of the same precursor m/z. Dynamic exclusion was enabled with a mass exclusion time of 3 min and a repeat count of 1 within 30 sec of initial m/z measurement. Spectra were collected over the entirety of each 90 minute chromatography run.

Identification of peptides

Raw mass spectra were converted to MGF format using MSConvert, part of the ProteoWizard software library (36), in preparation for spectrum-database matching. The mass spectra for each culture type were searched against the reference proteome of *M. hyorhinis* HUB-1 (12) using X!tandem 2015.12.15.2 (37) and OMSSA 2.1.8 (38) algorithms. Because MDCK and McCoy cell line proteins are impossible to completely remove from isolated *Mycoplasma* cells using the techniques described above, NCBI reference protein sets for *Canis familiaris* and *Mus musculus* were also included in the analysis. A fasta database was constructed by concatenating the HUB-1, MDCK, and McCoy protein fasta files into a single file. A randomized version of this database was then concatenated to create a combined database which could be used to identify peptides and evaluate dataset quality simultaneously. X!tandem and OMSSA algorithms were employed via the University of Arizona High Performance Computing

Center to perform spectrum matching. Precursor and fragment mass tolerance were set to 0.3 Daltons for both OMSSA and X!tandem. Trypsin cleavage rules were used for both algorithms with up to 2 missed cleavages. Amino acid modifications searched consisted of single and double oxidation of Methionine, oxidation of Proline, N-terminal acetylation, carbamidomethylation of Cysteine, deamidation of Asparagine and Glutamine and phosphorylation of Serine, Threonine, and Tyrosine. X!tandem xml and OMSSA xml results were filtered using the Perl programming language to remove any peptide matches with an E-value > 0.05 as well as proteins identified by a single peptide sequence. To address the problem of contaminating host proteins, only peptides uniquely identifying each protein were retained for further analysis. This ensured neither *Canis* nor *Mus* peptides affect the calculated expression levels for HUB-1 proteins.

Comparative protein analysis

Differential expression of proteins between broth cultured MHR and that grown in MDCK and McCoy cultures was evaluated using peptide elution profiles. Analyses were performed separately for broth vs. MDCK, broth vs. McCoy, and MDCK vs. McCoy comparisons. Precursor mass spectra were extracted from the raw data in MS1 format using the MSConvert GUI software from the ProteoWizard tool set (36). Peptide precursor m/z values were extracted from the previously compiled protein identifications using Perl. Elution profiles for peptide-spectrum matches were calculated by parsing each corresponding MS1 file and summing the ion current for that match's m/z value within a 0.3 Da tolerance, effectively integrating the elution profiles. Each trace started at the scan number of the peptide-spectrum match and preceded both forward and backward until the chromatogram noise level, or a distance of 250 scans, was reached. Multiple peptide-spectrum matches with the same precursor

m/z were only counted once, ensuring the same integral was not included multiple times. Once all peptide-spectrum matches were processed, intensities were summed for each protein on a per-replicate basis. Proteins not identified in a replicate were represented with the average noise level of the replicate's chromatogram for further calculations. The reasoning behind this is two-fold: 1) peptides not identified in a replicate could be present at levels at or below the noise level of the chromatogram causing the mass spectrometer to ignore them, and 2) for calculating expression ratios between lines, zero cannot be in the denominator. Data were normalized using a mode based technique. First, the mode of the protein intensities for each replicate was calculated, representing the most commonly occurring protein intensity. Next, for each identified protein, the intensity per replicate was divided by the mode of the same replicate. This ensures that normalization is not affected by the minimum and maximum intensities, which can vary tremendously between replicates. A resampling analysis was performed for each protein by evaluating the difference in means of the replicates of both conditions. From this resampling analysis, a *p*-value was calculated indicating the significance of the difference in means. Two additional resampling analyses were performed for each protein, comparing both conditions to their own baselines. These baseline analyses provide a mechanism to further reduce false positives introduced by differences in chromatogram ion current as electron multiplier performance decreases. Proteins were considered to be differentially expressed if the difference in means between conditions resulted in a p-value ≤ 0.05 and the difference in means between one of the conditions and its baseline was ≤ 0.05. Results of the differential expression analysis were saved as a tab-delimited text file.

NCBI gene identifiers for *M. hyorhinis* HUB-1 were used to retrieve other database information using the “Retrieve/ID mapping” tool of UniProt (14). Associated information for

these proteins, including KEGG identifiers, GO terms, gene names, and cross-reference identifiers, were retrieved from various databases. A candidate list of proteins significant for differential expression as described above was prepared for each comparison. Proteins from each list were used to draw an intersecting Venn diagram using BioVenn (39) in order to identify proteins that were overlapping between comparisons.

Authors' Contributions

BM performed lab culturing and authored the manuscript. FCM edited the manuscript. KP performed the sample preparation, mass spectrometry and spectra analyses and contributed to and edited the manuscript.

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CHAPTER 6. CONCLUSION

General Discussion

A challenge model for *Mycoplasma hyorhinis* (MHR) was developed for use in 6-7 week old caesarian-derived, colostrum-deprived (CDCD) pigs. The challenge material used in this model is derived from infected tissue culture. This is not the first mycoplasma model to utilize such material (1), nor is it the first MHR model to cause polyserositis and/or lameness (2-11). It is, however, the first model to use a) cell-associated MHR, b) in CDCD pigs, c) utilizing a consecutive-day administration, and d) resulting in severe and consistently reproducible outcomes of pericarditis, arthritis and lameness. The consecutive-day challenge administration was found to result in pericarditis and lameness in ≥ 50% of the challenged animals; whereas, administration by a single route (e.g. intraperitoneal, intravenous) on a single day resulted in only one of these outcomes within our observation period. Intravenous administration tended to result in a high incidence of polyserositis but low lameness. While intraperitoneal administration tended to result in more lameness but less polyserositis. A single intranasal application resulted in little-to-no disease. The consecutive three-day challenge model initially developed was refined to a two-day model, omitting the intranasal challenge while retaining similar disease outcomes.

The intranasal findings are a bit surprising. As MHR colonizes the upper respiratory tract, lateral and/or vertical transmission via the respiratory route was suspected (12-14). It is entirely possible that using our virulent, cell-associated material via the intranasal route may still cause disease; however, this administration may require more time for clinical signs to manifest. The 21-day post-challenge period in this model is both clinically efficient and economical with drastic clinical results. These are desired outcomes in models to be utilized

for vaccination-challenge studies as the length of these studies adds to increased overall costs for the investigator.

An experimental vaccine was developed for evaluation in the challenge model. This proprietary vaccine (Boehringer Ingelheim Vetmedica, Inc.) is a whole-cell bacterin consisting of MHR-infected murine fibroblasts and an oil-based adjuvant. Three doses of this vaccine (high, medium, and low) were evaluated for efficacy in three-week old CDCD piglets with primary parameters being pericarditis and lameness. Animals were vaccinated at three-weeks of age, challenged three weeks- post vaccination, and necropsied 21-days post challenge. All three vaccine doses provided for a significant reduction in pericarditis as compared to a placebo control group. The high and medium doses of vaccine also provided for significant reductions of lameness compared to the placebo control group. All vaccinated animals weighed significantly more than the placebo control group at the time of necropsy. Such a vaccine will provide benefit in reducing clinical signs of disease associated with MHR in the production setting and will further help to reduce antibiotic usage currently utilized for such.

An experiment was also conducted to evaluate the susceptibility to the challenge model in increasingly older animals. Groups of the same litters of CDCD pig were challenged with MHR at seven, ten, thirteen and sixteen weeks of age. Animals were necropsied 21-days post challenge. Animals showed an age-related decrease in outcomes of pericarditis, with nearly 90 % affected when challenged at seven weeks but slightly less than 30 % affected when challenged at ten weeks of age. This declining trend continued, ultimately resulting in just 4 % of the animals with pericarditis when challenged at sixteen weeks of age. Lameness, however, was similar in the seven, ten, and thirteen week of age

challenged groups with 65-68 % of the animals affected. It was not until the challenge at sixteen weeks of age that lameness dropped, with only 33 % of the animals being affected. These results will help define the desired duration of immunity for experimental MHR vaccines. This should also aid in the diagnostic setting; whereas the likelihood of MHR contributing to polyserositis declines rapidly from seven weeks of age and would therefore be an unlikely, or at most, a rare cause of polyserositis in animals three months of age and older.

Lastly, we performed a proteomic analysis between two *ex vivo* cultures of MHR and a conventionally derived broth culture and confirmed differential expression in the tissue culture-associated samples. While our data cannot confirm that the expression noted *ex vivo* more closely resembles that of MHR *in vivo* expression, several membrane-associated proteins of interest were identified and will be candidates for future research for associations with virulence and/or potential vaccine evaluations. Some of the identified proteins are very likely the reason both our cell-associated challenge and vaccine culture have shown greater success than our broth culture counterparts (data not shown).

These studies culminate in the design and successful generation of a safe and efficacious vaccine which will aid in the reduction of MHR-associated polyserositis, arthritis and lameness in pigs.

Recommendations for Future Research

To truly determine whether *ex vivo* cultures better approximate the proteome expressions of *in vivo* cultures of MHR, affected tissues from infected pigs need to be collected and analyzed in a manner similar to that described in Chapter 5. In fact, we have collected pericardial scrapings from MHR challenged and non-challenged animals for such

an experiment. Unfortunately, the sheer quantities of host protein present in the samples severely hindered peptide identification and the associated differential analysis (data not shown). Better sample collection methods or sample preparation techniques should be evaluated to achieve distinguishable MHR peptide sequences to allow for differential analysis to the *ex vivo* samples. Perhaps synovial fluid may be a "cleaner" sample with fewer host proteins to sort through. Further, accompanying transcriptomics (i.e. RNA-seq) would provide supportive data for confirmation of the quantity of mRNA present for translation in the samples. Haider and Pal have provided examples of combining the two approaches (15).

Similarly, as we have examined the effect an association with tissue culture has on the protein expression of MHR, one could conversely examine what effect the MHR is having on the genomic and/or proteomic expression in the tissue culture cells. Studies such as these may provide further insight into the mechanisms MHR employ to cause infection in a living cell.

The membrane proteins identified in our proteomic analysis provide a narrow list of candidates one could evaluate as subunit vaccines or in a vector-delivery system. These approaches have been evaluated for *M. hyopneumoniae*, although results were in either in non-target species or were met with varying results in the swine host (16-24). The "cocktail" approach suggested by Galli *et al* (17) may provide the best chance for positive results given the number and variability of MHR surface proteins. Regardless of approach, our research has provided for a model to evaluate them in and a whole-cell vaccine to use as a positive control to compare efficacy.

Alternatively, recent genetic advances may allow for evaluations of deletion mutants of MHR in the host animal utilizing the disease model described herein. Such evaluations

targeting proteins identified in our proteomic analysis could help to define virulence factors for this organism. A method for site-directed mutagenesis has been described for *M. hyopneumoniae* (25). The breakthrough with CRISPR (26, 27) has yet to be fully exploited for Mycoplasma species. It is hopeful such tools can adapted for use with *Mycoplasma hyorhinis*.

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APPENDIX A. SUPPLEMENTAL INFORMATION FOR CHAPTER 2

Table S1. Study 1 dose and route of administration on day of study.

Group	Day 0	Day 1	Day 2
1 (n=10)	40 mL IP* (1.59 x 10^9 CCU)	—	—
2 (n=10)	20 mL IV* (7.96 x 10^8 CCU)	—	—
3 (n=10)	20 mL IN* (7.96 x 10^8 CCU)	—	—
4 (n=10)	40 mL IP (1.59 x 10^9 CCU)	20 mL IV (8.53 x 10^8 CCU)	20 mL IN (4.18 x 10^8 CCU)
5 (n=10)	20 mL IP (7.96 x 10^8 CCU)	10 mL IV (4.27 x 10^8 CCU)	10 mL IN (2.09 x 10^8 CCU)
6 (n=6)	—	—	—

*IP = intraperitoneal, IV = intravenous, IN = intranasal.

Table S2. Study 2 dose and route of administration on day of study.

Group	Day 0	Day 1	Day 2
1 (n=28)	20 mL IP (1.12 x 10^9 CCU)	10 mL IV (6.76 x 10^8 CCU)	10 mL IN (1.0 x 10^8 CCU)
2 (n=28)	20 mL IP (1.12 x 10^9 CCU)	10 mL IV (6.76 x 10^8 CCU)	—
3 (n=8)	—	—	—

*IP = intraperitoneal, IV = intravenous, IN = intranasal.

Table S3. Scoring system for respiration, coughing and lameness

Observation	Score 0	Score 1	Score 2	Score 3	Score 4	Score 5
Respiration	Normal	Abnormal	—	—	—	—
Coughing	Normal	Abnormal	—	—	—	—
Lameness	Normal (no visible lameness)	Mild (not constantly lame when walking, walks at a normal speed, is weight bearing while walking and standing, lameness is indicated by intermittent reduced weight bearing on one limb or shortening of the stride)	Moderate (constant and observed throughout every step at a walking pace, bearing some weight on the leg at a walk and standing, but short-striding one or more legs while walking, walks at a normal speed)	Moderately Severe (non-weight bearing on the leg the first few steps after standing, constant, obvious lameness while at a walking pace, putting very little weight on the leg at a walk or while standing, requires the pig to slow its speed of walking)	Severe (will stand (may require assistance) for at least 3 minutes, non-weight bearing on one or more legs at walk or standing, still able to three-legged walk)	Recumbent (will not stand even with assistance)

Table S4. Schedule of Events for Study 1.

Time	**Event** (group)	Samples
Day 0	Challenge by IP (1, 4, 5) Challenge by IV (2) Challenge by IN (3)	Nasal swabs collected for PCR Blood collected for ELISA Weight measurement
Day 1	Challenge by IV (4, 5)	None
Day 2	Challenge by IN (4, 5)	None
Days 0-21	(all)	Clinical observations and Respiratory, Coughing, and Lameness Scores
Day 21	Euthanasia (all)	Gross examination for polyserositis Lung tissue collection (fresh for PCR, fixed for histopathology) Heart tissue collection (fresh for PCR, fixed for histopathology) Elbow/stifle joint swab for PCR Carpi/tarsi joint swab for PCR Weight measurement

*IP = intraperitoneal, IV = intravenous, IN = intranasal.

Table S5. Schedule of Events for Study 2.

Time	**Event** (group)	Samples
Day 0	Challenge by IP (1, 2)	Nasal swabs collected for PCR Blood collected for ELISA Weight measurement
Day 1	Challenge by IV (1, 2)	None
Day 2	Challenge by IN (1)	None
Days 0-28	(all)	Clinical observations and Respiratory, Coughing, and Lameness Scores
Day 28	Euthanasia (all)	Gross examination for polyserositis Pericardial swab for PCR Pleural swab for PCR Elbow/stifle joint swab for PCR Carpi/tarsi joint swab for PCR Weight measurement

*IP = intraperitoneal, IV = intravenous, IN = intranasal.

Table S6. Study 1 average daily gain (ADG) statistical summary.

Treatment Group	Number	Mean (kg/day)	Standard Error	Lower 95% CI	Upper 95% CI
1	10	0.5	0.05	0.4	0.7
2	9	0.5	0.05	0.4	0.6
3	10	0.7	0.03	0.6	0.7
4	10	0.5	0.05	0.3	0.6
5	10	0.4	0.05	0.3	0.5
6	6	0.7	0.04	0.6	0.8

Groups: 1=IP (intraperitoneal), 2=IV (intravenous), 3=IN (intranasal), 4=IP,IV,IN (high dose), 5=IP,IV,IN (low dose). One animal in Group 2 died prior to D21. CI=confidence interval.

Table S7. Study 1 polyserositis statistical summary.

Treatment Group	Ratio Postive/Total	Positive (%)	Standard Error	Lower 95% CI	Upper 95% CI
Pleuritis					
1	0/10	0	0	0	30.8
2	3/10	30	14.5	6.7	65.2
3	0/10	0	0	0	30.8
4	4/10	40	15.5	12.2	73.8
5	2/10	20	12.6	2.5	55.6
6	0/6	0	0	0	45.9
Peritonitis					
1	0/10	0	0	0	30.8
2	1/10	10	9.5	0.3	44.5
3	0/10	0	0	0	30.8
4	0/10	0	0	0	30.8
5	0/10	0	0	0	30.8
6	0/6	0	0	0	45.9
Pericarditis					
1	2/10	20	12.6	2.5	55.6
2	9/10	90	9.5	55.5	99.7
3	0/10	0	0	0	30.8
4	5/10	50	15.8	18.7	81.3
5	5/10	50	15.8	18.7	81.3
6	0/6	0	0	0	45.9
Pleuritis or Peritonitis or Pericarditis Positive					
1	2/10	20	12.6	2.5	55.6
2	9/10	90	9.5	55.5	99.7
3	0/10	0	0	0	30.8
4	6/10	60	15.5	26.2	87.8
5	5/10	50	15.8	18.7	81.3
6	0/6	0	0	0	45.9

Groups: 1=IP (intraperitoneal), 2=IV (intravenous), 3=IN (intranasal), 4=IP,IV,IN (high dose), 5=IP,IV,IN (low dose). CI=confidence interval (Clopper-Pearson exact confidence limits).

Table S8. Study 1 arthritis statistical summary.

Treatment Group	Ratio Postive/Total	Positive (%)	Standard Error	Lower 95% CI	Upper 95% CI
1	3/10	30	14.5	6.7	65.2
2	1/10	10	9.5	0.3	44.5
3	1/10	10	9.5	0.3	44.5
4	5/10	50	15.8	18.7	81.3
5	5/10	50	15.8	18.7	81.3
6	0/6	0	0	0	45.9

Groups: 1=IP (intraperitoneal), 2=IV (intravenous), 3=IN (intranasal), 4=IP,IV,IN (high dose), 5=IP,IV,IN (low dose). CI=confidence interval (Clopper-Pearson exact confidence limits). A positive animal showed at least one joint with signs of arthritis.

Table S9. Study 1 PCR results statistical summary.

Treatment Group	Ratio Postive/Total	Positive (%)	Standard Error	Lower 95% CI	Upper 95% CI
Heart Tissue					
1	1/10	10	9.5	0.3	44.5
2	3/10	30	14.5	6.7	65.2
3	0/10	0	0	0	30.8
4	3/10	30	14.5	6.7	65.2
5	1/10	10	9.5	0.3	44.5
6	0/6	0	0	0	45.9
Lung Tissue					
1	1/10	10	9.5	0.3	44.5
2	3/10	30	14.5	6.7	65.2
3	0/10	0	0	0	30.8
4	4/10	40	15.5	12.2	73.8
5	1/10	10	9.5	0.3	44.5
6	0/6	0	0	0	45.9
Carpal/Tarsal Swab					
1	2/10	20	12.6	2.5	55.6
2	1/10	10	9.5	0.3	44.5
3	1/10	10	9.5	0.3	44.5
4	0/10	0	0	0	30.8
5	0/10	0	0	0	30.8
6	0/6	0	0	0	45.9
Elbow/Stifle Swab					
1	0/10	0	0	0	30.8
2	2/10	20	12.6	2.5	55.6
3	0/10	0	0	0	30.8
4	0/10	0	0	0	30.8
5	0/10	0	0	0	30.8
6	0/6	0	0	0	45.9

Groups: 1=IP (intraperitoneal), 2=IV (intravenous), 3=IN (intranasal), 4=IP,IV,IN (high dose), 5=IP,IV,IN (low dose). CI=confidence interval (Clopper-Pearson exact confidence limits).

Table S10. Study 1 lameness statistical summary.

Treatment Group	Ratio Postive/Total	Positive (%)	Standard Error	Lower 95% CI	Upper 95% CI
1	4/10	40	15.5	12.2	73.8
2	1/10	10	9.5	0.3	44.5
3	1/10	10	9.5	0.3	44.5
4	1/10	10	9.5	0.3	44.5
5	7/10	70	14.5	34.8	93.3
6	0/6	0	0	0	45.9

Groups: 1=IP (intraperitoneal), 2=IV (intravenous), 3=IN (intranasal), 4=IP,IV,IN (high dose), 5=IP,IV,IN (low dose). CI=confidence interval (Clopper-Pearson exact confidence limits). A pig was considered positive if it received a score ≥ 1 for any two consecutive days.

Table S11. Study 1 Histology statistical summary.

Treatment Group	Ratio Postive/Total	Positive (%)	Standard Error	Lower 95% CI	Upper 95% CI
Pleuritis of the Lungs					
1	0/10	0	0	0	30.8
2	2/9	22.2	13.9	2.8	60
3	0/10	0	0	0	30.8
4	2/10	20	12.6	2.5	55.6
5	3/10	30	14.5	6.7	65.2
6	0/6	0	0	0	45.9
Peribronchiolar/Septal Infiltrates of the Lungs					
1	3/10	30	14.5	6.7	65.2
2	5/9	55.6	16.6	21.2	86.3
3	0/10	0	0	0	30.8
4	4/10	40	15.5	12.2	73.8
5	4/10	40	15.5	12.2	73.8
6	0/6	0	0	0	45.9
Lymphocytic Inflammation of the Heart					
1	1/10	10	9.5	0.3	44.5
2	6/9	66.7	15.7	29.9	92.5
3	0/10	0	0	0	30.8
4	3/10	30	14.5	6.7	65.2
5	5/10	50	15.8	18.7	81.3
6	0/6	0	0	0	45.9
Fibrosis of the Heart					
1	3/10	30	14.5	6.7	65.2
2	8/9	88.9	10.5	51.8	99.7
3	0/10	0	0	0	30.8
4	4/10	40	15.5	12.2	73.8
5	5/10	50	15.8	18.7	81.3
6	0/6	0	0	0	45.9

Groups: 1=IP (intraperitoneal), 2=IV (intravenous), 3=IN (intranasal), 4=IP,IV,IN (high dose), 5=IP,IV,IN (low dose). CI=confidence interval (Clopper-Pearson exact confidence limits). A pig was considered positive if it received a score >0 for each respective category. One animal in Group 2 died prior to D21 and fixed tissue was not collected.

Table S12. Study 2 average daily gain (ADG) statistical summary.

Treatment group	Number	Least Squares Means	Standard Error	Lower 95% CI	Upper 95% CI	*p*-value
1	27	0.56	0.031	0.43	0.69	0.2991
2	28	0.51	0.031	0.38	0.64	

Groups: 1=IP,IV,IN, 2=IP,IV. IP=intraperitoneal; IV=intravenous; IN=intranasal. One animal in Group 1 died prior to D28. Analyses performed between challenged groups; non-infected group 3 was housed separately and not included.

Table S13. Study 2 polyserositis statistical summary.

Treatment group	Number	Mean	Standard Error of Mean	Lower 95% CI	Upper 95% CI	*p*-value
Pleuritis						
1	28	0.178	0.1063	0.000	1.000	0.6471
2	28	0.098	0.0737	0.000	1.000	
Pericarditis						
1	28	0.571	0.0979	0.008	0.995	0.4072
2	28	0.75	0.0848	0.010	0.999	

Groups: 1=IP,IV,IN, 2=IP,IV. IP=intraperitoneal; IV=intravenous; IN=intranasal. Mean=back transformed least squares means (proportion positive). No peritonitis was observed. Analyses performed between challenged groups; non-infected group 3 was housed separately and not included.

Table S14. Study 2 PCR results statistical summary.

Treatment group	Number	Mean	Standard Error of Mean	Lower 95% CI	Upper 95% CI	*p*-value
Pericardial Swab						
1	28	0.225	0.0976	0.000	0.997	0.9720
2	28	0.220	0.0970	0.000	0.997	
Pleural Swab						
1	28	0.143	0.0664	0.000	0.994	0.6142
2	28	0.214	0.0780	0.001	0.990	
Carpal/Tarsal Swab						
1	28	0.608	0.0975	0.009	0.996	0.4523
2	28	0.754	0.0848	0.009	0.999	
Elbow/Stifle Swab						
1	28	0.534	0.1295	0.002	0.999	0.6553
2	28	0.640	0.1217	0.002	0.999	

Groups: 1=IP,IV,IN, 2=IP,IV.. Mean=back transformed least squares means (proportion positive). Analyses performed between challenged groups; non-infected group 3 was housed separately and not included.

Table S15. Study 2 arthritis statistical summary.

Treatment group	Number	Mean	Standard Error of Mean	Lower 95% CI	Upper 95% CI	*p*-value
Left Elbow						
1	28	0.143	0.0661	0.000	0.994	0.4218
2	28	0.286	0.0854	0.002	0.988	
Left Stifle						
1	28	0.107	0.0584	0.000	0.996	0.4774
2	28	0.214	0.0776	0.001	0.990	
Left Tarsal						
1	28	0.182	0.0860	0.000	0.997	0.4089
2	28	0.343	0.1172	0.001	0.997	
Right Elbow						
1	28	0.088	0.0777	0.000	1.000	0.5192
2	228	0.239	0.1506	0.000	1.000	
Right Stifle						
1	28	0.105	0.0584	0.000	0.997	0.7519
2	28	0.140	0.0669	0.000	0.995	
Right Tarsal						
1	28	0.262	0.1039	0.000	0.997	0.6058
2	28	0.354	0.1183	0.001	0.997	
Any Joint Positive for Arthritis						
1	28	0.346	0.1063	0.001	0.995	0.4608
2	28	0.500	0.1141	0.003	0.997	

Groups: 1=IP,IV,IN, 2=IP,IV. IP=intraperitoneal; IV=intravenous; IN=intranasal. Mean=back transformed least squares means (proportion positive). Carpi from both groups remained free from arthritis. Analyses performed between challenged groups; non-infected group 3 was housed separately and not included.

Table S16. Study 2 clinical observations statistical summary.

Treatment group	Number	Mean	Standard Error of Mean	Lower 95% CI	Upper 95% CI	*p*-value
Respiration						
1	28	0.644	0.1036	0.006	0.998	0.7057
2	28	0.714	0.0963	0.006	0.999	
Coughing						
1	28	0.077	0.1253	0.000	1.000	0.9308
2	28	0.059	0.1007	0.000	1.000	
Lameness						
1	28	0.670	0.1096	0.004	0.999	0.7683
2	28	0.620	0.1151	0.003	0.999	

Groups: 1=IP,IV,IN, 2=IP,IV. IP=intraperitoneal; IV=intravenous; IN=intranasal. Mean=back transformed least squares means (proportion positive). A pig was positive for respiration or coughing if ever not normal. A pig was positive for lameness if it received a score of ≥ 1 on any two consecutive days. Analyses performed between challenged groups; non-infected group 3 was housed separately and not included.

APPENDIX B. SUPPLEMENTAL INFORMATION FOR CHAPTER 3

Table S17. Challenge dose and route of administration on day of study.

Group	0 dpc*	1 dpc	2 dpc	Total Applied Dose
1 (n=24)	Day 0: 20 mL/IP**	Day 1: 10 mL/IV**	Day 2: 10 mL/IN**	5.73×10^9 CCU***/pig
2 (n=25)	Day 21: 20 mL/IP	Day 22: 10 mL/IV	Day 23: 10 mL/IN	2.55×10^9 CCU/pig
3 (n=25)	Day 42: 20 mL/IP	Day 43: 10 mL/IV	Day 44: 10 mL/IN	3.72×10^9 CCU/pig
4 (n=25)	Day 63: 20 mL/IP	Day 64: 10 mL/IV	Day 65: 10 mL/IN	4.26×10^9 CCU/pig
5-8 (n=5, each)	—	—	—	—

*dpc = days post-challenge; **IP = intraperitoneal, IV = intravenous, IN = intranasal; ***CCU = color changing units.

Table S18. Schedule of Events.

Time	Event	Group	Samples
-1 dpc*	Sample collection	All	Blood collection for ELISA Nasal swab collection for PCR
0 dpc	Challenge by IP**	1, 2, 3, 4	Weight measurement
1 dpc	Challenge by IV**	1, 2, 3, 4	None
2 dpc	Challenge by IN**	1, 2, 3, 4	None
0 dpc - 21 dpc	Observations	All	Clinical observations and lameness scoring
21 dpc	Euthanasia	All	Gross examination for polyserositis Pericardial swab for PCR Pleural swab for PCR (if pleuritis present) Peritoneal swab for PCR (if peritonitis present) Elbow/stifle joint swab for PCR Carpal/tarsal joint swab for PCR Weight measurement

*dpc = days post challenge; **IP = intraperitoneal, IV = intravenous, IN = intranasal. Group: **1** = 7 weeks of age (0 dpc = Day 0); **2** = 10 weeks of age (0 dpc = Day 21); **3** = 13 weeks of age (0 dpc = Day 42); **4** = 16 weeks of age (0 dpc = Day 63).

Table S19. Statistical summaries for weight measurements.

Treatment Group*	Number	Mean (lbs)	Standard Error	Lower 95% CI	Upper 95% CI
0 dpc					
1	23	22.0	0.67	20.6	23.4
2	25	50.8	1.02	48.7	52.9
3	25	93.5	1.87	89.7	97.4
4	24	125.7	2.24	121.0	130.3
5	5	22.8	0.80	20.5	25.0
6	5	51.0	4.21	39.3	62.7
7	5	98.7	2.98	90.4	107.0
8	5	132.7	5.45	117.6	147.9
21 dpc					
1	22	32.0	1.36	29.2	34.8
2	24	69.6	1.76	66.0	73.3
3	23	114.9	2.44	109.9	120.0
4	23	160.3	2.88	154.3	166.3
5	5	50.0	1.65	45.4	54.5
6	5	87.3	5.64	71.6	102.9
7	5	145.1	5.79	129.0	161.1
8	5	181.0	6.65	162.6	199.5
Average Daily Gain (ADG)					
1	22	0.5	0.06	0.4	0.6
2	24	0.9	0.08	0.7	1.1
3	23	1.0	0.06	0.9	1.1
4	23	1.7	0.12	1.4	1.9
5	5	1.3	0.08	1.1	1.5
6	5	1.7	0.08	1.5	1.9
7	5	2.2	0.17	1.7	2.7
8	5	2.3	0.13	1.9	2.7

***1 and 5** = 7 weeks of age (0 dpc = Day 0); **2 and 6** = 10 weeks of age (0 dpc = Day 21); **3 and 7** = 13 weeks of age (0 dpc = Day 42); **4 and 8** = 16 weeks of age (0 dpc = Day 63). CI=confidence interval, dpc = days post challenge.

Table S20. Statistical summaries for polyserositis.

Treatment Group*	Number	Proportion Positive	Standard Error	Lower 95% CI	Upper 95% CI
Pericarditis					
1	23	0.870	0.070	0.664	0.972
2	25	0.280	0.090	0.121	0.494
3	25	0.080	0.054	0.010	0.260
4	24	0.042	0.041	0.001	0.211
5	5	0.000	0.000	0.000	0.522
6	5	0.000	0.000	0.000	0.522
7	5	0.000	0.000	0.000	0.522
8	5	0.000	0.000	0.000	0.522
Pleuritis					
1	23	0.261	0.092	0.102	0.484
2	25	0.040	0.039	0.001	0.204
3	25	0.160	0.073	0.045	0.361
4	24	0.000	0.000	0.000	0.142
5	5	0.000	0.000	0.000	0.522
6	5	0.000	0.000	0.000	0.522
7	5	0.000	0.000	0.000	0.522
8	5	0.000	0.000	0.000	0.522
Peritonitis					
1	23	0.217	0.086	0.075	0.437
2	25	0.160	0.073	0.045	0.361
3	25	0.080	0.054	0.010	0.260
4	24	0.000	0.000	0.000	0.142
5	5	0.000	0.000	0.000	0.522
6	5	0.000	0.000	0.000	0.522
7	5	0.000	0.000	0.000	0.522
8	5	0.000	0.000	0.000	0.522

***1 and 5** = 7 weeks of age (0 dpc = Day 0); **2 and 6** = 10 weeks of age (0 dpc = Day 21); **3 and 7** = 13 weeks of age (0 dpc = Day 42); **4 and 8** = 16 weeks of age (0 dpc = Day 63). CI=confidence interval.

Table S21. Statistical summaries for *M. hyorhinis* PCR.

Treatment Group*	Number	Proportion Positive	Standard Error	Lower 95% CI	Upper 95% CI
Pericardial Swabs					
1	23	0.261	0.092	0.102	0.484
2	25	0.040	0.039	0.001	0.204
3	25	0.000	0.000	0.000	0.137
4	24	0.083	0.056	0.010	0.270
5	5	0.000	0.000	0.000	0.522
6	5	0.000	0.000	0.000	0.522
7	5	0.000	0.000	0.000	0.522
8	5	0.000	0.000	0.000	0.522
Pleural Swabs					
1	6	0.333	0.192	0.043	0.777
2	1	0.000	0.000	0.000	0.975
3	4	0.000	0.000	0.000	0.602
Peritoneal Swabs					
1	5	0.200	0.179	0.005	0.716
2	4	0.000	0.000	0.000	0.602
3	2	0.000	0.000	0.000	0.842
Elbow/Stifle Swabs					
1	23	0.826	0.079	0.612	0.950
2	25	0.360	0.096	0.180	0.575
3	25	0.320	0.093	0.149	0.535
4	24	0.167	0.076	0.047	0.374
5	5	0.000	0.000	0.000	0.522
6	5	0.000	0.000	0.000	0.522
7	5	0.000	0.000	0.000	0.522
8	5	0.000	0.000	0.000	0.522
Carpal/Tarsal Swabs					
1	23	0.870	0.070	0.664	0.972
2	25	0.360	0.096	0.180	0.575
3	25	0.120	0.065	0.025	0.312
4	24	0.042	0.041	0.001	0.211
5	5	0.000	0.000	0.000	0.522
6	5	0.000	0.000	0.000	0.522
7	5	0.000	0.000	0.000	0.522
8	5	0.000	0.000	0.000	0.522

***1 and 5** = 7 weeks of age (0 dpc = Day 0); **2 and 6** = 10 weeks of age (0 dpc = Day 21); **3 and 7** = 13 weeks of age (0 dpc = Day 42); **4 and 8** = 16 weeks of age (0 dpc = Day 63). CI=confidence interval.

Table S22. Statistical summaries for arthritis.

Group	Number	Proportion Positive	Standard Error	Lower 95% CI	Upper 95% CI
At least one joint positive					
1	23	1.000	0.000	0.852	1.000
2	25	0.560	0.099	0.349	0.756
3	25	0.240	0.085	0.094	0.451
4	24	0.250	0.088	0.098	0.467
5	5	0.000	0.000	0.000	0.522
6	5	0.000	0.000	0.000	0.522
7	5	0.000	0.000	0.000	0.522
8	5	0.000	0.000	0.000	0.522
Proportion joints positive per animal					
1	23	0.462	0.047	0.365	0.559
2	25	0.175	0.041	0.091	0.259
3	25	0.040	0.016	0.008	0.072
4	24	0.089	0.036	0.015	0.162
5	5	0.000	0.000	.	.
6	5	0.000	0.000	.	.
7	5	0.000	0.000	.	.
8	5	0.000	0.000	.	.

***1 and 5** = 7 weeks of age (0 dpc = Day 0); **2 and 6** = 10 weeks of age (0 dpc = Day 21); **3 and 7** = 13 weeks of age (0 dpc = Day 42); **4 and 8** = 16 weeks of age (0 dpc = Day 63). CI=confidence interval.

Table S23. Statistical summary for lameness.

Treatment Group*	Number	Proportion Positive	Standard Error	Lower 95% CI	Upper 95% CI
1	23	0.652	0.099	0.427	0.836
2	25	0.680	0.093	0.465	0.851
3	25	0.680	0.093	0.465	0.851
4	24	0.333	0.096	0.156	0.553
5	5	0.000	0.000	0.000	0.522
6	5	0.200	0.179	0.005	0.716
7	5	0.000	0.000	0.000	0.522
8	5	0.400	0.219	0.053	0.853

***1 and 5** = 7 weeks of age (0 dpc = Day 0); **2 and 6** = 10 weeks of age (0 dpc = Day 21); **3 and 7** = 13 weeks of age (0 dpc = Day 42); **4 and 8** = 16 weeks of age (0 dpc = Day 63). CI=confidence interval. A pig was considered positive if it received a score ≥ 1 for any two consecutive days.

APPENDIX C. SUPPLEMENTAL INFORMATION FOR CHAPTER 4

Table S24. Summary of observations for abnormal respirations and coughing.

Treatment[1]	Affected animals/total animals	Mean[2]	Standard Error	PF[3] vs CTRL	Lower 95% CI[4]	Upper 95% CI
CTRL	5/26	0.1947	0.0821	—	—	—
HIGH	1/28	0.0346	0.0345	0.82	-0.63	0.98
MED	3/27	0.1085	0.0614	0.44	-1.30	0.86
LOW	4/26	0.1510	0.0729	0.22	-1.78	0.78

1 = CTRL—placebo control, HIGH—high dose vaccine, MED—medium dose vaccine, LOW—low dose vaccine; NTX group not included in analysis. Two CTRL pigs were excluded from analysis due to lameness at the time of challenge.
2 = least squares mean
3 = preventive fraction
4 = confidence interval

Table S25. Summary of observations for lameness.

Treatment[1]	Affected animals/total animals	Mean[2]	Standard Error	PF[3] vs CTRL	Lower 95% CI[4]	Upper 95% CI
CTRL	14/26	0.5402	0.1302	—	—	—
HIGH	6/28	0.1922	0.0897	0.64	0.10	0.86
MED	4/27	0.1287	0.0727	0.76	0.26	0.92
LOW	6/26	0.2200	0.0993	0.59	-0.01	0.84

1 = CTRL—placebo control, HIGH—high dose vaccine, MED—medium dose vaccine, LOW—low dose vaccine; NTX group not included in analysis. Two CTRL pigs were excluded from analysis due to lameness at the time of challenge.
2 = least squares mean
2 = preventive fraction
3 = confidence interval

Table S26. Summary of incidence of pericarditis.

Treatment[1]	Affected animals/total animals	Mean[2]	Standard Error	PF[3] vs CTRL	Lower 95% CI[4]	Upper 95% CI
CTRL	25/26	0.9615	0.03771	—	—	—
HIGH	13/28	0.4643	0.09425	0.52	0.26	0.69
MED	11/27	0.4074	0.09456	0.58	0.31	0.74
LOW	17/26	0.6538	0.0933	0.32	0.07	0.5

1 = CTRL—placebo control, HIGH—high dose vaccine, MED—medium dose vaccine, LOW—low dose vaccine; NTX group not included in analysis. Two CTRL pigs were excluded from analysis due to lameness at the time of challenge.
2 = least squares mean
2 = preventive fraction
3 = confidence interval

Table S27. Summary of weight gain in the vaccination phase and challenge phase.

Treatment[1]	Number of Animals	Mean[2] (lbs)	Standard Error	*p*-value	Lower 95% CI[4]	Upper 95% CI
Day 21 (Vaccination Phase)						
CTRL	26	14.58	0.61	—	—	—
HIGH	28	14.21	0.59	0.5490	-0.86	1.60
MED	27	13.90	0.60	0.2820	-0.57	1.93
LOW	28	13.67	0.60	0.1440	-0.32	2.14
Day 42 (Challenge Phase)						
CTRL	25	26.62	1.32	—	—	—
HIGH	28	34.45	1.27	< 0.0001	-11.30	-4.38
MED	27	33.12	1.29	< 0.0001	-9.99	-3.03
LOW	26	30.56	1.29	0.0270	-7.43	-0.46

1 = CTRL—placebo control, HIGH—high dose vaccine, MED—medium dose vaccine, LOW—low dose vaccine; NTX group not included in analysis. Two CTRL pigs were excluded from analysis due to lameness at the time of challenge.
2 = least squares mean
2 = preventive fraction
3 = confidence interval

Table S28. Summary of average daily weight gain (ADG).

Treatment[1]	Number of Animals	Mean[2]	Standard Error	*p*-value	Lower 95% CI[4]	Upper 95% CI
CTRL	25	0.57	0.05	—	—	—
HIGH	28	0.96	0.05	< 0.0001	-0.53	-0.25
MED	27	0.92	0.05	< 0.0001	-0.48	-0.21
LOW	26	0.80	0.05	0.0012	-0.37	-0.09

1 = CTRL—placebo control, HIGH—high dose vaccine, MED—medium dose vaccine, LOW—low dose vaccine; NTX group not included in analysis. Two CTRL pigs were excluded from analysis due to lameness at the time of challenge.
2 = least squares mean
2 = preventive fraction
3 = confidence interval

APPENDIX D. SUPPLEMENTAL INFORMATION FOR CHAPTER 5

Table S29. Identification of 34 proteins unique to the Broth vs. McCoy comparison and significant ($p < 0.05$) for differential expression. Protein identification by liquid chromatography, tandem mass spectrometry (LC-MS/MS) using *M. hyorhinis* HUB-1 database.

UniProtKB Entry[1]	Protein Description[2]	Gene Name[3]	Fold Change[4] Broth vs. McCoy
E0TK92	ABC transporter permease protein	MHR_0064	8.59
E0TKG7	hypothetical protein	MHR_0453	8.58
E0TLN4	hypothetical protein	MHR_0655	8.36
E0TL22	Phosphotransferase enzyme family protein	MHR_0211	8.03
E0TKC7	DNA polymerase III gamma and tau subunit	(*dnaX*) MHR_0101	7.83
E0TKA1	P59-like protein	MHR_0073	5.74
E0TL86	Glutamyl-tRNA amidotransferase subunit B	(*gatB*) MHR_0573	2.77
E0TLE9	Fatty acid-binding protein DegV-like protein	MHR_0265	2.68
E0TL73	Elongation factor Tu	(*tuf*) MHR_0560	-1.21
E0TK65	NADH oxidase	(*nox*) MHR_0035	-1.27
E0TL32	ATP synthase subunit alpha	(*atpA*) MHR_0221	-1.32
E0TL34	Proton-translocating ATPase, beta subunit	(*atpD*) MHR_0223	-1.78
E0TLD6	hypothetical protein	MHR_0628	-2.04
E0TK50	Glycyl tRNA synthetase	(*glyS*) MHR_0410	-2.24
E0TLG9	hypothetical protein	MHR_0285	-2.42
E0TK39	50S ribosomal protein L2	(*rplB*) MHR_0399	-2.59
E0TLA4	Ribosome recycling factor	(*frr*) MHR_0595	-3.12
E0TLJ6	Methyltransferase type 11	(*cdd*) MHR_0312	-3.28
E0TKU9	NH(3)-dependent NAD(+) synthetase	(*nadE*) MHR_0509	-6.39
E0TKU8	hypothetical protein	MHR_0508	-7.13
E0TKW4	Predicted GTPase, probable translation factor	(*gtp1*) MHR_0524	-7.31
E0TK46	Probable endonuclease 4	(*nfo*) MHR_0406	-7.33
E0TK34	50S ribosomal protein L29	(*rmpC*) MHR_0394	-7.58
E0TL26	putative acyl carrier protein	MHR_0215	-7.70
E0TLM8	TrkA-C domain protein	(*trkA*) MHR_0649	-8.25
E0TL76	uracil phosphoribosyltransferase	(*upp*) MHR_0563	-8.84
E0TK04	Oligopeptide transport system permease protein	(*oppF*) MHR_0359	-8.93
E0TLH7	hypothetical protein	MHR_0293	-9.06
E0TK28	30S ribosomal protein S8	(*rpsH*) MHR_0388	-9.07
E0TLD8	hypothetical protein	MHR_0630	-9.18
E0TK31	50S ribosomal protein L24	(*rplX*) MHR_0391	-9.20
E0TLI1	COF family HAD hydrolase protein	MHR_0297	-9.61

Table S29 (continued)

E0TKJ1	Phosphotransferase system (PTS) enzyme I	(*ptsI*) MHR_0477	-9.91
E0TK02	Lipoprotein	MHR_0357	-10.46

[1] unique entry identifier in the UniProtKB database (www.uniprot.org)
[2] names and synonyms of the protein in the UniProtKB database (www.uniprot.org)
[3] *Mycoplasma hyorhinis* HUB-1 gene name according to NCBI database (www.ncbi.nlm.nih.gov)
[4] Fold change of second term as compared to first term, expressed as log2

Table S30. Identification of 20 proteins unique to the Broth vs. MDCK comparison and significant ($p < 0.05$) for differential expression. Protein identification by liquid chromatography, tandem mass spectrometry (LC-MS/MS) using *M. hyorhinis* HUB-1 database.

UniProtKB Entry[1]	Protein Description[2]	Gene Name[3]	Fold Change[4] Broth vs. MDCK
E0TJZ5	Glycerol-3-phosphate dehydrogenase [NAD(P)+]	(*gpsA*) MHR_0350	9.88
E0TLF5	50S ribosomal protein L11	(*rplK*) MHR_0271	9.25
E0TKA2	Amino acid or sugar ABC transport system, permease protein	MHR_0074	9.05
E0TL87	Putative transcriptional regulator	MHR_0574	8.70
E0TJW1	Putative MgpA-like protein	MHR_0006	8.66
E0TLP1	Ribonucleoside-diphosphate reductase beta chain	(*nrdF*) MHR_0662	8.54
E0TL98	LemA-family protein	MHR_0589	2.85
E0TLB9	hypothetical 33 kDa chaperonin	MHR_0610	2.81
E0TK08	Oligoendopeptidase F	(*pepF*) MHR_0363	2.64
E0TLN2	ATP synthase subunit B	(*atpD*) MHR_0653	2.52
E0TKG9	Probable L-ribulose-5-phosphate 3-epimerase, UlaE	(*sgaU*) MHR_0455	2.50
E0TKL0	Ribose-phosphate pyrophosphokinase	(*prsA*) MHR_0117	1.69
E0TLF7	CTP synthase	(*pyrG*) MHR_0273	1.61
E0TLM3	hypothetical protein	MHR_0644	1.60
E0TLJ3	Phosphopentomutase	(*deoB*) MHR_0309	1.35
E0TKI3	Enolase	(*eno*) MHR_0469	0.84
E0TKE5	L-lactate dehydrogenase	(*ldh*) MHR_0431	-0.90
E0TK36	30S ribosomal protein S3	(*rpsC*) MHR_0396	-1.66
E0TLN8	Lipoprotein	MHR_0659	-2.66
E0TKQ7	Xylose ABC transporter permease protein	(*xylH*) MHR_0164	-9.85

[1] unique entry identifier in the UniProtKB database (www.uniprot.org)
[2] names and synonyms of the protein in the UniProtKB database (www.uniprot.org)
[3] *Mycoplasma hyorhinis* HUB-1 gene name according to NCBI database (www.ncbi.nlm.nih.gov)
[4] Fold change of second term as compared to first term, expressed as log2

Table S31. Identification of 22 proteins unique to the MDCK vs. McCoy comparison and significant ($p < 0.05$) for differential expression. Protein identification by liquid chromatography, tandem mass spectrometry (LC-MS/MS) using *M. hyorhinis* HUB-1 database.

UniProtKB Entry[1]	Protein Description[2]	Gene Name[3]	Fold Change[4] MDCK vs. McCoy
E0TKW7	30S ribosomal protein S6	(*rpsF*) MHR_0527	7.36
E0TLE4	Oligopeptide transport system permease protein	(*oppC*) MHR_0637	2.53
E0TKV4	pyruvate dehydrogenase E3 component dihydrolipoamide dehydrogenase	(*pdhD*) MHR_0514	-0.63
E0TLK0	FMN-dependent NADH-azoreductase	(*acpD*) MHR_0316	-1.27
E0TK51	Signal recognition particle protein	(*ffh*) MHR_0411	-1.52
E0TKV9	50S ribosomal protein L20	(*rplT*) MHR_0519	-1.53
E0TKY6	6-phosphofructokinase	(*pfkA*) MHR_0547	-1.62
E0TKI6	Thiol-disulfide isomerase and thioredoxin	(*trxA*) MHR_0472	-1.65
E0TK52	Elongation factor Ts	(*tsf*) MHR_0412	-1.69
E0TKK1	ABC transporter xylose-binding lipoprotein	MHR_0487	-2.04
E0TLE3	Oligopeptide ABC transporter ATP binding protein	(*oppD*) MHR_0636	-2.08
E0TLK3	ABC transporter ATP-binding protein	MHR_0319	-2.16
E0TLQ8	Membrane protein, OxaA	(*oxaA*) MHR_0679	-2.17
E0TKT8	Protein P115	(*smc*) MHR_0498	-2.27
E0TKT5	Predicted kinase, related to dihydroxyacetone kinase	MHR_0495	-2.35
E0TKH7	VACB-like ribonuclease II	(*rnr*) MHR_0463	-2.39
E0TK30	50S ribosomal protein L5	(*rplE*) MHR_0390	-2.79
E0TKK2	ABC transporter, ATP-binding protein	(*ecfA*) MHR_0109	-5.60
E0TLL2	Expressed protein	MHR_0328	-5.99
E0TLF1	ATP-dependent helicase, PcrA	(*pcrA*) MHR_0267	-7.70
E0TLB0	Putative ATP-binding helicase protein	MHR_0601	-7.70
E0TLH8	hypothetical protein	MHR_0294	-9.24

[1] unique entry identifier in the UniProtKB database (www.uniprot.org)
[2] names and synonyms of the protein in the UniProtKB database (www.uniprot.org)
[3] *Mycoplasma hyorhinis* HUB-1 gene name according to NCBI database (www.ncbi.nlm.nih.gov)
[4] Fold change of second term as compared to first term, expressed as log2

Table S32. Identification of overlapping proteins common to all three comparisons (Broth vs. McCoy, Broth vs. MDCK, and MDCK vs. McCoy) and significant ($p < 0.05$) for differential expression. Protein identification by liquid chromatography, tandem mass spectrometry (LC-MS/MS) using *M. hyorhinis* HUB-1 database.

UniProtKB Entry[1]	Protein Description[2]	Gene Name[3]	Fold Change[4] Broth vs. MDCK	Fold Change[4] Broth vs. McCoy	Fold Change[4] MDCK vs. McCoy
E0TLD9	Chromosome replication initiation and membrane attachment protein	MHR_0632	6.49	9.15	2.66
E0TK90	uncharactized lipoprotein	MHR_0062	2.59	1.66	-0.92
E0TL96	Neutrophil activating factor	(*napA*) MHR_0587	1.78	-7.41	-9.19
E0TLB8	Trigger factor	(*tig*) MHR_0609	0.71	-0.94	-1.65
E0TL16	aminopeptidase	MHR_0205	-1.29	-2.81	-1.51
E0TL59	101 kDa protein	MHR_0249	-1.80	-9.99	-8.20
E0TK40	50S ribosomal protein L23	(*rplW)* MHR_0400	-2.11	-10.54	-8.43
E0TK82	50S ribosomal protein L7/L12	(*rplL*) MHR_0054	-2.45	-11.46	-9.01
E0TL36	101 kDa protein	MHR_0225	-11.88	-2.99	8.89

[1] unique entry identifier in the UniProtKB database (www.uniprot.org)
[2] names and synonyms of the protein in the UniProtKB database (www.uniprot.org)
[3] *Mycoplasma hyorhinis* HUB-1 gene name according to NCBI database (www.ncbi.nlm.nih.gov)
[4] Fold change of second term as compared to first term, expressed as log2

Table S33. Identification of 29 overlapping proteins common to both Broth vs. *ex vivo* comparisons (Broth vs. McCoy and Broth vs. MDCK) and significant ($p < 0.05$) for differential expression. Protein identification by liquid chromatography, tandem mass spectrometry (LC-MS/MS) using *M. hyorhinis* HUB-1 database.

UniProtKB Entry[1]	Protein Description[2] (Enzyme class)	Gene Name[3]	Fold Change[4] Broth vs. MDCK	Fold Change[4] Broth vs. McCoy
E0TKB6	Arginyl-tRNA synthetase 1	(*argS*) MHR_0090	9.16	8.48
E0TKM4	Topoisomerase IV subunit A	(*parC*) MHR_0131	8.97	7.80
E0TKZ3	putative intracellular protease/amidase	MHR_0554	7.76	7.32
E0TKW8	adenine phosphoribosyltransferase	(*apt*) MHR_0528	3.26	2.78
E0TK89	uncharactized lipoprotein	MHR_0061	2.04	2.59
E0TKQ1	Serine hydroxymethyltransferase 3	(*glyA*) MHR_0158	1.84	2.09
E0TKQ6	xylose ABC transporter ATP-binding protein	(*xylG*) MHR_0163	-2.36	-1.32
E0TK18	DNA-directed RNA polymerase subunit alpha	(*rpoA*) MHR_0377	-0.83	-1.62
E0TL54	Transcription antitermination protein	(*nusG*) MHR_0243	-1.63	-1.77
E0TK01	Oligoendopeptidase F	(*pepF*) MHR_0356	-1.29	-1.79
E0TLC1	molecular chaperone DnaK	(*dnaK*) MHR_0612	-1.73	-2.39
E0TK27	50S ribosomal protein L6	(*rplF*) MHR_0387	-2.12	-3.06
E0TK03	hypothetical protein	MHR_0358	-3.91	-3.29
E0TKY9	ATP-dependent serine proteinase, heat shock protein	(*clpB*) MHR_0550	-2.61	-3.84
E0TK32	50S ribosomal protein L14	(*rplN*) MHR_0392	-8.08	-7.71
E0TKF4	Outer membrane protein-P95	MHR_0440	-8.46	-8.09
E0TKD7	Ribulose-phosphate 3-epimerase	(*rpe*) MHR_0423	-8.49	-8.12
E0TKF8	hypothetical protein	MHR_0444	-8.51	-8.14
E0TKR0	hypothetical protein	MHR_0167	-8.69	-8.32
E0TLH0	hypothetical protein	MHR_0286	-8.71	-8.34
E0TKZ2	Zinc metalloproteinase C	MHR_0553	-8.94	-8.58
E0TK17	50S ribosomal protein L17	(*rplQ*) MHR_0376	-9.17	-8.80
E0TK35	50S ribosomal protein L16	(*rplP*) MHR_0395	-9.23	-8.86
E0TLN9	hypothetical protein	MHR_0660	-9.27	-8.90
E0TK38	30S ribosomal protein S19	(*rpsS*) MHR_0398	-9.32	-8.95
E0TKF3	hypothetical protein	MHR_0439	-9.47	-9.10
E0TKF7	Excinuclease ATPase subunit-like protein	(*uvrA*) MHR_0443	-9.88	-9.51
E0TKJ2	50S ribosomal protein L21	(*rplU*) MHR_0478	-9.91	-9.54
E0TLJ8	hypothetical protein	MHR_0314	-10.76	-10.39

[1] unique entry identifier in the UniProtKB database (www.uniprot.org)
[2] names and synonyms of the protein in the UniProtKB database (www.uniprot.org)
[3] *Mycoplasma hyorhinis* HUB-1 gene name according to NCBI database (www.ncbi.nlm.nih.gov)
[4] Fold change of second term as compared to first term, expressed as log2

Table S34. McCoy culture *M. hyorhinis* proteins

NCBI GI	NCBI Accession	UniProt Accession	Protein Description	% Coverage	Unique Spectra	Unique Peptides
304373392	YP_003856601.1	E0TLC0	Glyceraldehyde 3-phosphate dehydrogenase C	81.38	229	21
304373258	YP_003856467.1	E0TKI6	Thiol-disulfide isomerase and thioredoxin	79.21	30	7
304373351	YP_003856560.1	E0TL79	Probable purine nucleoside phosphorylase transmembrane protein	77.59	127	12
304373302	YP_003856511.1	E0TKV6	Pyruvate dehydrogenase E1 component beta subunit	74.09	136	21
304373345	YP_003856554.1	E0TL73	Elongation factor Tu	67.91	179	20
304372893	YP_003856102.1	E0TKB8	Fructose-bisphosphate aldolase class II transmembrane protein	62.15	46	9
304373314	YP_003856523.1	E0TKW8	adenine phosphoribosyltransferase	59.78	44	11
304373300	YP_003856509.1	E0TKV4	pyruvate dehydrogenase E3 component dihydrolipoamide dehydrogenase	58.35	121	23
304372963	YP_003856172.1	E0TKQ5	hypothetical protein MHR_0162	57.72	118	22
304373217	YP_003856426.1	E0TKE5	L-lactate dehydrogenase	57.19	127	15
304373112	YP_003856321.1	E0TLK0	FMN-dependent NADH-azoreductase	55.72	35	10
304373349	YP_003856558.1	E0TL77	Deoxyribose-phosphate aldolase 1	55.61	32	8
304372864	YP_003856073.1	E0TK89	uncharactized lipoprotein	54.99	90	31
304372872	YP_003856081.1	E0TK97	Histone-like DNA-binding protein	54.44	9	3
304373303	YP_003856512.1	E0TKV7	Pyruvate dehydrogenase E1-alpha subunit	53.55	111	16
304373393	YP_003856602.1	E0TLC1	molecular chaperone DnaK	52.43	128	22
304372959	YP_003856168.1	E0TKQ1	Serine hydroxymethyltransferase 3	52.39	43	12
304373222	YP_003856431.1	E0TKF0	Phosphoglycerate kinase	51.00	47	14
304373255	YP_003856464.1	E0TKI3	Enolase	50.89	152	18
304373409	YP_003856618.1	E0TLD7	membrane protease subunits, stomatin/prohibitin-like protein, partial	50.00	13	6
304373097	YP_003856306.1	E0TLI5	Acetate kinase AckA	49.5	30	14
304373246	YP_003856455.1	E0TKH4	hypothetical protein MHR_0460	48.86	25	11
304373096	YP_003856305.1	E0TLI4	Phosphate acetyltransferase	48.74	42	10
304372835	YP_003856044.1	E0TK60	aminopeptidase	48.48	69	17
304373432	YP_003856641.1	E0TLN2	ATP synthase subunit B	47.84	35	14
304372957	YP_003856166.1	E0TKP9	Triosephosphate isomerase	47.33	38	9
304373113	YP_003856322.1	E0TLK1	FMN-dependent NADH-azoreductase 1	46.39	73	6
304373016	YP_003856225.1	E0TL29	ATP synthase C chain, sodium ion specific lipid-binding protein	45.87	20	3
304373068	YP_003856277.1	E0TLF6	50S ribosomal protein L1	44.78	16	7
304373200	YP_003856409.1	E0TK52	Elongation factor Ts	44.59	27	8
304373370	YP_003856579.1	E0TL98	LemA-family protein	44.35	23	5

Table S34 (continued)

304373201	YP_003856410.1	E0TK53	30S ribosomal protein S2	43.71	28	11
304373166	YP_003856375.1	E0TK18	DNA-directed RNA polymerase subunit alpha	40.84	23	9
304372890	YP_003856099.1	E0TKB5	Heat shock protein	40.38	22	8
304373187	YP_003856396.1	E0TK39	50S ribosomal protein L2	39.86	24	7
304372908	YP_003856117.1	E0TKD3	Hypoxanthine phosphoribosyltransferase	39.56	8	5
304372840	YP_003856049.1	E0TK65	NADH oxidase	39.52	35	14
304373021	YP_003856230.1	E0TL34	Proton-translocating ATPase, beta subunit	39.15	34	10
304373350	YP_003856559.1	E0TL78	Thymidine phosphorylase	38.52	23	9
304373308	YP_003856517.1	E0TKW2	Thiol peroxidase	35.47	28	4
304373358	YP_003856567.1	E0TL86	Glutamyl-tRNA amidotransferase subunit B	35.02	25	11
304373086	YP_003856295.1	E0TLH4	30S ribosomal protein S16	34.44	2	2
304372813	YP_003856022.1	E0TJW3	dihydrolipoamide dehydrogenase	34.34	33	13
304373332	YP_003856541.1	E0TKY6	6-phosphofructokinase	33.44	21	9
304373190	YP_003856399.1	E0TK42	50S ribosomal protein L3	33.33	10	6
304373156	YP_003856365.1	E0TK08	Oligoendopeptidase F	33.22	22	13
304373241	YP_003856450.1	E0TKG9	Probable L-ribulose-5-phosphate 3-epimerase ulaE	32.66	25	7
304373390	YP_003856599.1	E0TLB8	Trigger factor	32.44	60	14
304372918	YP_003856127.1	E0TKL0	Ribose-phosphate pyrophosphokinase	32.21	14	7
304372949	YP_003856158.1	E0TKP1	Xaa-pro aminopeptidase	32.01	16	8
304373158	YP_003856367.1	E0TK10	Probable transketolase transmembrane protein	31.54	35	10
304373243	YP_003856452.1	E0TKH1	Pentitol phosphotransferase enzyme II, A component	31.41	7	3
304372865	YP_003856074.1	E0TK90	uncharactized lipoprotein	31.21	44	19
304373339	YP_003856548.1	E0TKZ3	putative intracellular protease/amidase	30.94	5	3
304373292	YP_003856501.1	E0TKU6	Pyruvate kinase	29.68	45	11
304373129	YP_003856338.1	E0TLL7	Nicotinate phosphoribosyltransferase	28.96	17	6
304372899	YP_003856108.1	E0TKC4	Lipoyltransferase and lipoate-protein ligase	28.88	14	8
304372900	YP_003856109.1	E0TKC5	Expressed protein	28.85	3	2
304372952	YP_003856161.1	E0TKP4	Seryl-trna synthetase protein	28.67	11	6
304373175	YP_003856384.1	E0TK27	50S ribosomal protein L6	28.49	8	3
304373433	YP_003856642.1	E0TLN3	ATP synthase subunit A	28.40	27	13
304373184	YP_003856393.1	E0TK36	30S ribosomal protein S3	27.98	12	5
304373275	YP_003856484.1	E0TKS9	Translation initiation factor IF-2	27.81	25	11
304373312	YP_003856521.1	E0TKW6	Single-stranded DNA-binding protein	27.37	7	3
304373380	YP_003856589.1	E0TLA8	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	27.29	16	8
304373146	YP_003856355.1	E0TJZ8	cell division protein FtsZ	27.27	16	7

Table S34 (continued)

304373178	YP_003856387.1	E0TK30	50S ribosomal protein L5	27.07	8	3
304373032	YP_003856241.1	E0TL45	50S ribosomal protein L9	26.90	7	4
304373415	YP_003856624.1	E0TLE3	Oligopeptide ABC transporter ATP binding prote	26.55	9	5
304373105	YP_003856314.1	E0TLJ3	Phosphopentomutase	26.01	14	7
304373425	YP_003856634.1	E0TLM5	Pyrophosphatephospho hydrolase	25.54	6	4
304373041	YP_003856250.1	E0TL54	Transcription antitermination protein	24.75	11	4
304373301	YP_003856510.1	E0TKV5	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	24.68	16	9
304373144	YP_003856353.1	E0TJZ6	Protein mraZ	24.49	6	3
304372903	YP_003856112.1	E0TKC8	hypothetical protein MHR_0102	24.21	2	2
304373288	YP_003856497.1	E0TKU2	translation elongation factor G	23.64	19	10
304373414	YP_003856623.1	E0TLE2	Oligopeptide transport system permease protein	23.6	20	13
304372886	YP_003856095.1	E0TKB1	30S ribosomal protein S4	23.41	9	4
304373240	YP_003856449.1	E0TKG8	Sugar isomerase SgaE	23.36	5	4
304372898	YP_003856107.1	E0TKC3	Triacylglycerol lipase	22.85	11	5
304373108	YP_003856317.1	E0TLJ6	Methyltransferase type 11	22.66	3	2
304373019	YP_003856228.1	E0TL32	ATP synthase subunit alpha	22.44	19	10
304373282	YP_003856491.1	E0TKT6	Phosphate acyltransferase	22.36	7	4
304373066	YP_003856275.1	E0TLF4	Putative asparaginyl-trna synthetase protein	22.20	26	7
304373084	YP_003856293.1	E0TLH2	50S ribosomal protein L19	21.67	4	2
365897173	YP_004956719.1	E4PYV0	dihydrofolate reductase	21.38	4	3
304372852	YP_003856061.1	E0TK77	5'-nucleotidase	21.07	26	9
304373189	YP_003856398.1	E0TK41	50S ribosomal protein L4	21.02	10	5
304373418	YP_003856627.1	E0TLE6	Lipoprotein	20.82	37	15
304373167	YP_003856376.1	E0TK19	30S ribosomal protein S13	20.49	2	2
304373431	YP_003856640.1	E0TLN1	hypothetical protein MHR_0652	20.00	7	5
304373405	YP_003856614.1	E0TLD3	High affinity transport system protein p37	19.76	13	7
304372933	YP_003856142.1	E0TKM5	Isoleucyl tRNA synthetase	19.73	17	12
304372925	YP_003856134.1	E0TKL7	50S ribosomal protein L13	19.31	5	2
304372964	YP_003856173.1	E0TKQ6	xylose ABC transporter ATP-binding protein	19.29	16	10
304373020	YP_003856229.1	E0TL33	ATP synthase gamma chain	19.15	9	3
304373227	YP_003856436.1	E0TKF5	Thioredoxin reductase	19.02	6	4
304373331	YP_003856540.1	E0TKY5	hypothetical protein MHR_0546	18.84	14	6
304373199	YP_003856408.1	E0TK51	Signal recognition particle protein	18.65	11	5
304373242	YP_003856451.1	E0TKH0	hexulose 6 phosphate synthase	18.3	12	2

Table S34 (continued)

304373317	YP_003856526.1	E0TKX1	Lysyl-tRNA synthetase 1	18.29	8	4
304372915	YP_003856124.1	E0TKK7	DNA gyrase subunit A	18.24	21	10
304372891	YP_003856100.1	E0TKB6	Arginyl-tRNA synthetase 1	18.01	11	6
304372876	YP_003856085.1	E0TKA1	P59-like protein	17.58	19	7
304373259	YP_003856468.1	E0TKI7	prolyl-tRNA synthetase	17.5	10	5
304373058	YP_003856267.1	E0TL71	Glycerophosphodiester phosphodiesterase family protein	17.43	5	3
304373006	YP_003856215.1	E0TL19	5'-nucleotidase	16.71	8	4
304372859	YP_003856068.1	E0TK84	DNA-directed RNA polymerase subunit beta'	16.55	36	15
304373073	YP_003856282.1	E0TLG1	Lipoprotein	16.47	15	7
304373271	YP_003856480.1	E0TKJ9	DNA polymerase I	16.22	8	3
304373067	YP_003856276.1	E0TLF5	50S ribosomal protein L11	16.00	2	2
304373334	YP_003856543.1	E0TKY8	Membrane nuclease, lipoprotein	15.96	12	5
304373149	YP_003856358.1	E0TK01	Oligoendopeptidase F	15.93	21	6
304372807	YP_003856016.1	E0TJV7	DNA polymerase III beta subunit	15.78	9	4
304373010	YP_003856219.1	E0TL23	GTPase obg	15.55	6	4
304373174	YP_003856383.1	E0TK26	50S ribosomal protein L18	15.45	4	2
304372836	YP_003856045.1	E0TK61	molecular chaperone DnaJ	15.32	3	2
304372996	YP_003856205.1	E0TL09	Lipoprotein	15.28	17	10
304373221	YP_003856430.1	E0TKE9	P3	15.27	10	5
304373407	YP_003856616.1	E0TLD5	P60-like lipoprotein	14.65	12	6
304373323	YP_003856532.1	E0TKX7	alanyl-tRNA synthetase	14.54	14	9
304372879	YP_003856088.1	E0TKA4	Ag 243-5 protein	14.32	12	6
304373305	YP_003856514.1	E0TKV9	50S ribosomal protein L20	14.29	3	2
304373157	YP_003856366.1	E0TK09	Elongation factor P	13.98	2	2
304373423	YP_003856632.1	E0TLM3	hypothetical protein MHR_0644	13.70	8	6
304373143	YP_003856352.1	E0TJZ5	Glycerol-3-phosphate dehydrogenase [NAD(P)+]	13.51	4	3
304373029	YP_003856238.1	E0TL42	Fructose permease IIC component	13.47	11	6
304373376	YP_003856585.1	E0TLA4	Ribosome recycling factor	13.44	4	3
304373416	YP_003856625.1	E0TLE4	Oligopeptide transport system permease protein	13.28	9	3
304373061	YP_003856270.1	E0TLE9	Fatty acid-binding protein DegV-like protein	13.15	13	2
304373318	YP_003856527.1	E0TKX2	cell division protease FtsH	13.09	20	9
304373366	YP_003856575.1	E0TL94	ABC transporter ATP-binding protein	13.01	12	5
304373009	YP_003856218.1	E0TL22	Phosphotransferase enzyme family protein	12.86	5	3
304373270	YP_003856479.1	E0TKJ8	putative hydrolase	12.69	2	2
304373313	YP_003856522.1	E0TKW7	30S ribosomal protein S6	12.09	5	2

Table S34 (continued)

304373347	YP_003856556.1	E0TL75	hypothetical protein MHR_0562	11.74	2	2
304373118	YP_003856327.1	E0TLK6	Glutamyl-tRNA synthetase	11.73	7	4
304373436	YP_003856645.1	E0TLN6	hypothetical protein MHR_0657	11.73	4	2
304373408	YP_003856617.1	E0TLD6	hypothetical protein MHR_0628	11.63	12	7
304373359	YP_003856568.1	E0TL87	Putative transcriptional regulator	11.60	5	4
304373411	YP_003856620.1	E0TLD9	Chromosome replication initiation and membrane attachment protein	11.59	6	3
304373076	YP_003856285.1	E0TLG4	lipoate-protein ligase A	11.56	6	3
304373417	YP_003856626.1	E0TLE5	Oligopeptide ABC transporter permease protein	11.40	4	3
304373069	YP_003856278.1	E0TLF7	CTP synthase	11.38	12	4
304373372	YP_003856581.1	E0TLA0	Glucose-6-phosphate isomerase A	11.28	6	3
304373003	YP_003856212.1	E0TL16	aminopeptidase	11.08	8	5
304373081	YP_003856290.1	E0TLG9	hypothetical protein MHR_0285	10.88	14	6
304373335	YP_003856544.1	E0TKY9	ATP-dependent serine proteinase, heat shock protein	10.72	12	4
304373239	YP_003856448.1	E0TKG7	hypothetical protein MHR_0453	10.27	8	3
304373116	YP_003856325.1	E0TLK4	Peptide chain release factor 1	10.25	5	3
304372862	YP_003856071.1	E0TK87	hypothetical protein MHR_0059	10.21	3	2
304373284	YP_003856493.1	E0TKT8	Protein P115	10.21	19	7
304372946	YP_003856155.1	E0TKN8	aspartyl tRNA synthetase	9.95	6	4
304372811	YP_003856020.1	E0TJW1	Putative MgpA-like protein	9.54	5	3
304373045	YP_003856254.1	E0TL58	DNA gyrase subunit B	9.53	6	3
304373173	YP_003856382.1	E0TK25	30S ribosomal protein S5	9.42	5	2
304372945	YP_003856154.1	E0TKN7	Histidyl-tRNA synthetase	9.36	3	3
304372877	YP_003856086.1	E0TKA2	Amino acid or sugar ABC transport system, permease protein	9.28	7	5
304373296	YP_003856505.1	E0TKV0	mannose-6-phosphate isomerase	9.18	3	2
304373346	YP_003856555.1	E0TL74	ATP-dependent protease La	8.82	12	6
304372967	YP_003856176.1	E0TKQ9	Glucokinase	8.75	3	2
304373443	YP_003856652.1	E0TLP3	ribonucleotide-diphosphate reductase subunit alpha	8.74	10	4
304373030	YP_003856239.1	E0TL43	Leucyl-trna synthetase protein	8.50	10	5
304373277	YP_003856486.1	E0TKT1	Transcription termination-antitermination factor nusA	8.44	8	3
304373441	YP_003856650.1	E0TLP1	Ribonucleoside-diphosphate reductase beta chai	8.43	3	2
304373147	YP_003856356.1	E0TJZ9	hypothetical protein MHR_0354	8.11	4	2
304373031	YP_003856240.1	E0TL44	Predicted signaling protein	7.98	8	3
304373391	YP_003856600.1	E0TLB9	hypothetical 33 kDa chaperonin	7.93	5	3
304373286	YP_003856495.1	E0TKU0	30S ribosomal protein S12	7.91	4	2
304373056	YP_003856265.1	E0TL69	Glycerol-3-phosphate dehydrogenase, putative	7.79	3	3

Table S34 (continued)

304373434	YP_003856643.1	E0TLN4	hypothetical protein MHR_0655	7.71	9	5
304373153	YP_003856362.1	E0TK05	Oligopeptide transport system permease protein	7.69	3	2
304373198	YP_003856407.1	E0TK50	Glycyl tRNA synthetase	7.42	5	3
304373458	YP_003856667.1	E0TLQ8	Membrane protein oxaA	7.19	7	3
304372889	YP_003856098.1	E0TKB4	Heat-inducible transcription repressor hrcA	7.10	3	2
304373273	YP_003856482.1	E0TKK1	ABC transporter xylose-binding lipoprotein	7.02	2	2
304373385	YP_003856594.1	E0TLB3	Threonyl-tRNA synthetase	6.54	5	5
304372867	YP_003856076.1	E0TK92	ABC transporter permease protein	6.50	9	6
304373115	YP_003856324.1	E0TLK3	ABC transporter ATP-binding protein	6.43	4	2
304372873	YP_003856082.1	E0TK98	GTP-binding protein LepA	6.34	2	2
304373365	YP_003856574.1	E0TL93	tRNA modification GTPase mnmE	6.31	3	2
304373352	YP_003856561.1	E0TL80	Protein translocase subunit secA	6.30	11	4
304373281	YP_003856490.1	E0TKT5	Predicted kinase, related to dihydroxyacetone kinase	5.88	4	2
304373218	YP_003856427.1	E0TKE6	Hexosephosphate transport protein	5.81	8	2
304373438	YP_003856647.1	E0TLN8	Lipoprotein	5.58	7	4
304372931	YP_003856140.1	E0TKM3	Topoisomerase IV subunit B	5.24	4	2
304372858	YP_003856067.1	E0TK83	DNA-directed RNA polymerase subunit beta	4.77	8	5
304373057	YP_003856266.1	E0TL70	hypothetical protein MHR_0260	4.59	4	3
304373456	YP_003856665.1	E0TLQ6	Lipoprotein	4.52	3	2
304373151	YP_003856360.1	E0TK03	hypothetical protein MHR_0358	4.50	15	7
304372932	YP_003856141.1	E0TKM4	Topoisomerase IV subunit A	4.09	3	3
304373249	YP_003856458.1	E0TKH7	VACB-like ribonuclease II	4.03	2	2
304372806	YP_003856015.1	E0TJV6	Chromosomal replication initiator protein dnaA	3.47	2	2
304373114	YP_003856323.1	E0TLK2	ABC transporter permease protein	3.47	9	7
304372902	YP_003856111.1	E0TKC7	DNA polymerase III gamma and tau subunit	3.23	4	2
304372965	YP_003856174.1	E0TKQ7	Xylose ABC transporter permease protein	3.19	3	2
304373023	YP_003856232.1	E0TL36	101 kDa protein	2.86	6	2
304372845	YP_003856054.1	E0TK70	hypothetical protein MHR_0042	2.73	3	2
304372829	YP_003856038.1	E0TJX9	hypothetical protein MHR_0024	2.68	2	2
304373367	YP_003856576.1	E0TL95	hypothetical Y+L amino acid transporter 1	2.27	4	3
304373357	YP_003856566.1	E0TL85	Glutamyl-tRNA amidotransferase subunit A	1.75	4	3

Table S35. MDCK culture *M. hyorhinis* proteins

NCBI GI	NCBI Accession	UniProt Accession	Protein Description	% Coverage	Unique Spectra	Unique Peptides
304373345	YP_003856554.1	E0TL73	Elongation factor Tu	69.90	351	23
304373112	YP_003856321.1	E0TLK0	FMN-dependent NADH-azoreductase	68.16	318	12
304372893	YP_003856102.1	E0TKB8	Fructose-bisphosphate aldolase class II transmembrane protein	66.67	97	11
304372963	YP_003856172.1	E0TKQ5	hypothetical protein MHR_0162	65.55	511	24
304373258	YP_003856467.1	E0TKI6	Thiol-disulfide isomerase and thioredoxin	64.36	84	5
304373314	YP_003856523.1	E0TKW8	adenine phosphoribosyltransferase	64.25	111	10
304373405	YP_003856614.1	E0TLD3	High affinity transport system protein p37	60.71	110	20
304373244	YP_003856453.1	E0TKH2	Pentitol phosphotransferase enzyme II, B component	60.00	42	4
304373409	YP_003856618.1	E0TLD7	membrane protease subunits, stomatin/prohibitin-like protein, partial	60.00	20	6
304373302	YP_003856511.1	E0TKV6	Pyruvate dehydrogenase E1 component beta subunit	59.76	184	18
304373166	YP_003856375.1	E0TK18	DNA-directed RNA polymerase subunit alpha	57.36	97	14
304373392	YP_003856601.1	E0TLC0	Glyceraldehyde 3-phosphate dehydrogenase C	57.36	275	18
304373255	YP_003856464.1	E0TKI3	Enolase	56.89	289	23
304373113	YP_003856322.1	E0TLK1	FMN-dependent NADH-azoreductase 1	56.19	140	8
304373086	YP_003856295.1	E0TLH4	30S ribosomal protein S16	54.44	10	4
304372835	YP_003856044.1	E0TK60	aminopeptidase	54.35	196	20
304373217	YP_003856426.1	E0TKE5	L-lactate dehydrogenase	53.35	260	14
304373300	YP_003856509.1	E0TKV4	pyruvate dehydrogenase E3 component dihydrolipoamide dehydrogenase	52.46	181	26
304373303	YP_003856512.1	E0TKV7	Pyruvate dehydrogenase E1-alpha subunit	52.19	151	17
304372926	YP_003856135.1	E0TKL8	30S ribosomal protein S9	51.52	29	4
304373097	YP_003856306.1	E0TLI5	Acetate kinase AckA	51.49	81	16
304373016	YP_003856225.1	E0TL29	ATP synthase C chain, sodium ion specific lipid-binding protein	51.38	70	3
304372957	YP_003856166.1	E0TKP9	Triosephosphate isomerase	51.03	30	11
304373393	YP_003856602.1	E0TLC1	molecular chaperone DnaK	50.25	291	24
304373108	YP_003856317.1	E0TLJ6	Methyltransferase type 11	50.00	8	5
304373390	YP_003856599.1	E0TLB8	Trigger factor	49.49	184	23
304372925	YP_003856134.1	E0TKL7	50S ribosomal protein L13	48.97	9	5
304373067	YP_003856276.1	E0TLF5	50S ribosomal protein L11	48.67	9	4
304373308	YP_003856517.1	E0TKW2	Thiol peroxidase	48.26	15	5
304373187	YP_003856396.1	E0TK39	50S ribosomal protein L2	47.69	38	9
304373351	YP_003856560.1	E0TL79	Probable purine nucleoside phosphorylase transmembrane protein	47.41	48	11

Table S35 (continued)

304373349	YP_003856558.1	E0TL77	Deoxyribose-phosphate aldolase 1	45.74	27	7
365897170	YP_004956716.1	E4PYU7	transcription elongation factor	45.12	12	5
304373432	YP_003856641.1	E0TLN2	ATP synthase subunit B	44.83	64	12
304373174	YP_003856383.1	E0TK26	50S ribosomal protein L18	44.72	29	6
304373178	YP_003856387.1	E0TK30	50S ribosomal protein L5	44.20	41	6
304373021	YP_003856230.1	E0TL34	Proton-translocating ATPase, beta subunit	42.55	43	13
304373292	YP_003856501.1	E0TKU6	Pyruvate kinase	41.89	97	15
304373191	YP_003856400.1	E0TK43	30S ribosomal protein S10	41.35	11	5
304373259	YP_003856468.1	E0TKI7	prolyl-tRNA synthetase	40.42	58	12
304373425	YP_003856634.1	E0TLM5	Pyrophosphatephospho hydrolase	40.22	27	6
304372857	YP_003856066.1	E0TK82	50S ribosomal protein L7/L12	39.84	13	5
304373096	YP_003856305.1	E0TLI4	Phosphate acetyltransferase	38.99	49	10
304372890	YP_003856099.1	E0TKB5	Heat shock protein	38.85	34	10
304373200	YP_003856409.1	E0TK52	Elongation factor Ts	38.51	47	9
304373227	YP_003856436.1	E0TKF5	Thioredoxin reductase	38.03	44	10
304372872	YP_003856081.1	E0TK97	Histone-like DNA-binding protein	37.78	12	2
304373073	YP_003856282.1	E0TLG1	Lipoprotein	37.72	46	16
304373105	YP_003856314.1	E0TLJ3	Phosphopentomutase	37.63	45	10
304373301	YP_003856510.1	E0TKV5	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	37.50	28	8
304373006	YP_003856215.1	E0TL19	5'-nucleotidase	37.34	35	11
304373041	YP_003856250.1	E0TL54	Transcription antitermination protein	36.87	14	5
304373201	YP_003856410.1	E0TK53	30S ribosomal protein S2	36.23	36	9
304373222	YP_003856431.1	E0TKF0	Phosphoglycerate kinase	36.07	111	10
304372918	YP_003856127.1	E0TKL0	Ribose-phosphate pyrophosphokinase	35.89	48	9
304372865	YP_003856074.1	E0TK90	uncharactized lipoprotein	35.72	144	20
304373172	YP_003856381.1	E0TK24	50S ribosomal protein L15	35.14	9	5
304373246	YP_003856455.1	E0TKH4	hypothetical protein MHR_0460	34.86	16	10
304373185	YP_003856394.1	E0TK37	50S ribosomal protein L22	34.71	24	5
304372813	YP_003856022.1	E0TJW3	dihydrolipoamide dehydrogenase	34.34	118	14
304373370	YP_003856579.1	E0TL98	LemA-family protein	33.91	39	4
304373287	YP_003856496.1	E0TKU1	30S ribosomal protein S7	33.33	13	6
304373376	YP_003856585.1	E0TLA4	Ribosome recycling factor	33.33	7	3
304373068	YP_003856277.1	E0TLF6	50S ribosomal protein L1	33.04	31	9
304373056	YP_003856265.1	E0TL69	Glycerol-3-phosphate dehydrogenase, putative	32.47	17	8

Table S35 (continued)

304372864	YP_003856073.1	E0TK89	uncharactized lipoprotein	31.91	95	17
304373182	YP_003856391.1	E0TK34	50S ribosomal protein L29	31.88	4	2
304372959	YP_003856168.1	E0TKQ1	Serine hydroxymethyltransferase 3	31.82	40	11
304373243	YP_003856452.1	E0TKH1	Pentitol phosphotransferase enzyme II, A component	31.41	5	3
304373332	YP_003856541.1	E0TKY6	6-phosphofructokinase	30.96	49	9
304373350	YP_003856559.1	E0TL78	Thymidine phosphorylase	30.86	28	11
304373158	YP_003856367.1	E0TK10	Probable transketolase transmembrane protein	30.41	80	17
304373181	YP_003856390.1	E0TK33	30S ribosomal protein S17	30.00	11	3
304373443	YP_003856652.1	E0TLP3	ribonucleotide-diphosphate reductase subunit alpha	29.54	39	17
304373061	YP_003856270.1	E0TLE9	Fatty acid-binding protein DegV-like protein	29.41	11	5
304373176	YP_003856385.1	E0TK28	30S ribosomal protein S8	29.23	8	3
304373129	YP_003856338.1	E0TLL7	Nicotinate phosphoribosyltransferase	28.96	14	8
304372862	YP_003856071.1	E0TK87	hypothetical protein MHR_0059	28.87	16	5
304373175	YP_003856384.1	E0TK27	50S ribosomal protein L6	28.49	17	3
304373275	YP_003856484.1	E0TKS9	Translation initiation factor IF-2	28.31	43	11
304373380	YP_003856589.1	E0TLA8	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	28.27	23	10
304373242	YP_003856451.1	E0TKH0	hexulose 6 phosphate synthase	28.12	12	8
304373179	YP_003856388.1	E0TK31	50S ribosomal protein L24	28.04	4	2
304373305	YP_003856514.1	E0TKV9	50S ribosomal protein L20	27.73	17	4
304373407	YP_003856616.1	E0TLD5	P60-like lipoprotein	27.66	31	10
304373156	YP_003856365.1	E0TK08	Oligoendopeptidase F	27.63	44	12
304373339	YP_003856548.1	E0TKZ3	putative intracellular protease/amidase	27.62	8	3
304373436	YP_003856645.1	E0TLN6	hypothetical protein MHR_0657	27.55	5	4
304373003	YP_003856212.1	E0TL16	aminopeptidase	27.15	41	8
304372900	YP_003856109.1	E0TKC5	Expressed protein	26.92	4	2
304372949	YP_003856158.1	E0TKP1	Xaa-pro aminopeptidase	26.91	32	6
304373146	YP_003856355.1	E0TJZ8	cell division protein FtsZ	26.47	22	8
304373391	YP_003856600.1	E0TLB9	hypothetical 33 kDa chaperonin	26.44	28	8
304372840	YP_003856049.1	E0TK65	NADH oxidase	26.42	197	12
304373433	YP_003856642.1	E0TLN3	ATP synthase subunit A	26.23	29	8
304373049	YP_003856258.1	E0TL62	S-adenosylmethionine synthetase	25.79	24	8
304373190	YP_003856399.1	E0TK42	50S ribosomal protein L3	25.54	11	4
304373032	YP_003856241.1	E0TL45	50S ribosomal protein L9	25.52	5	3
304373189	YP_003856398.1	E0TK41	50S ribosomal protein L4	24.75	23	6
304372852	YP_003856061.1	E0TK77	5'-nucleotidase	24.68	44	12

Table S35 (continued)

304373281	YP_003856490.1	E0TKT5	Predicted kinase, related to dihydroxyacetone kinase	24.63	23	9
304373184	YP_003856393.1	E0TK36	30S ribosomal protein S3	24.28	17	5
304373240	YP_003856449.1	E0TKG8	Sugar isomerase SgaE	24.18	13	4
304373288	YP_003856497.1	E0TKU2	translation elongation factor G	24.07	85	13
304372964	YP_003856173.1	E0TKQ6	xylose ABC transporter ATP-binding protein	23.97	29	8
304373318	YP_003856527.1	E0TKX2	cell division protease FtsH	23.97	57	10
304373149	YP_003856358.1	E0TK01	Oligoendopeptidase F	23.32	51	11
304373428	YP_003856637.1	E0TLM8	TrkA-C domain protein	23.11	9	3
304373420	YP_003856629.1	E0TLM0	Ribose 5-phosphate isomerase B	22.88	7	3
304373418	YP_003856627.1	E0TLE6	Lipoprotein	22.62	86	16
304373312	YP_003856521.1	E0TKW6	Single-stranded DNA-binding protein	22.35	13	4
304373199	YP_003856408.1	E0TK51	Signal recognition particle protein	22.25	21	9
304372996	YP_003856205.1	E0TL09	Lipoprotein	22.00	44	13
304372891	YP_003856100.1	E0TKB6	Arginyl-tRNA synthetase 1	21.95	14	8
304373143	YP_003856352.1	E0TJZ5	Glycerol-3-phosphate dehydrogenase [NAD(P)+]	21.92	15	6
304372876	YP_003856085.1	E0TKA1	P59-like protein	21.68	14	7
304373167	YP_003856376.1	E0TK19	30S ribosomal protein S13	21.31	3	2
304373019	YP_003856228.1	E0TL32	ATP synthase subunit alpha	21.06	21	8
304373334	YP_003856543.1	E0TKY8	Membrane nuclease, lipoprotein	20.67	12	6
304373284	YP_003856493.1	E0TKT8	Protein P115	20.63	68	17
304373069	YP_003856278.1	E0TLF7	CTP synthase	20.55	29	8
304373408	YP_003856617.1	E0TLD6	hypothetical protein MHR_0628	20.36	26	10
304372858	YP_003856067.1	E0TK83	DNA-directed RNA polymerase subunit beta	20.15	58	15
304372952	YP_003856161.1	E0TKP4	Seryl-trna synthetase protein	20.14	13	5
304373188	YP_003856397.1	E0TK40	50S ribosomal protein L23	20.12	10	3
304372859	YP_003856068.1	E0TK84	DNA-directed RNA polymerase subunit beta'	20.00	62	20
304373029	YP_003856238.1	E0TL42	Fructose permease IIC component	19.48	14	9
304373320	YP_003856529.1	E0TKX4	Peptidyl-tRNA hydrolase	19.37	9	4
304373010	YP_003856219.1	E0TL23	GTPase obg	19.14	22	7
304373458	YP_003856667.1	E0TLQ8	Membrane protein oxaA	18.95	23	9
304373124	YP_003856333.1	E0TLL2	Expressed protein	18.91	8	3
304373173	YP_003856382.1	E0TK25	30S ribosomal protein S5	18.83	5	4
304372946	YP_003856155.1	E0TKN8	aspartyl tRNA synthetase	18.67	21	7
304373241	YP_003856450.1	E0TKG9	Probable L-ribulose-5-phosphate 3-epimerase ulaE	18.52	18	5
304373346	YP_003856555.1	E0TL74	ATP-dependent protease La	18.21	33	11

Table S35 (continued)

304373013	YP_003856222.1	E0TL26	putative acyl carrier protein	18.06	6	2
304373116	YP_003856325.1	E0TLK4	Peptide chain release factor 1	18.01	11	5
304372903	YP_003856112.1	E0TKC8	hypothetical protein MHR_0102	17.89	2	2
304373017	YP_003856226.1	E0TL30	ATP synthase subunit b	17.46	9	2
304373441	YP_003856650.1	E0TLP1	Ribonucleoside-diphosphate reductase beta chai	17.44	11	4
304372807	YP_003856016.1	E0TJV7	DNA polymerase III beta subunit	17.38	13	7
304373335	YP_003856544.1	E0TKY9	ATP-dependent serine proteinase, heat shock protein	16.99	20	7
304373385	YP_003856594.1	E0TLB3	Threonyl-tRNA synthetase	16.35	17	7
304373204	YP_003856413.1	E0TK56	Probable 1-acyl-sn-glycerol-3-phosphate acyltransferase	16.34	9	3
304373414	YP_003856623.1	E0TLE2	Oligopeptide transport system permease protein	16.3	27	8
304373066	YP_003856275.1	E0TLF4	Putative asparaginyl-trna synthetase protein	16.14	15	5
304373353	YP_003856562.1	E0TL81	deoxycytidylate deaminase	16.05	11	2
304373282	YP_003856491.1	E0TKT6	Phosphate acyltransferase	16.01	6	4
304373286	YP_003856495.1	E0TKU0	30S ribosomal protein S12	15.83	4	2
304373415	YP_003856624.1	E0TLE3	Oligopeptide ABC transporter ATP binding prote	15.82	15	6
304372879	YP_003856088.1	E0TKA4	Ag 243-5 protein	15.64	75	6
304372886	YP_003856095.1	E0TKB1	30S ribosomal protein S4	15.61	5	3
304373147	YP_003856356.1	E0TJZ9	hypothetical protein MHR_0354	15.48	12	4
304373431	YP_003856640.1	E0TLN1	hypothetical protein MHR_0652	15.12	16	4
304373331	YP_003856540.1	E0TKY5	hypothetical protein MHR_0546	14.91	10	6
304373221	YP_003856430.1	E0TKE9	P3	14.58	54	5
304373352	YP_003856561.1	E0TL80	Protein translocase subunit secA	14.57	17	11
304373145	YP_003856354.1	E0TJZ7	Ribosomal RNA small subunit methyltransferase H	14.43	5	2
304373435	YP_003856644.1	E0TLN5	hypothetical protein MHR_0656	14.38	7	4
304372811	YP_003856020.1	E0TJW1	Putative MgpA-like protein	14.15	5	3
304373366	YP_003856575.1	E0TL94	ABC transporter ATP-binding protein	14.13	26	6
304373045	YP_003856254.1	E0TL58	DNA gyrase subunit B	14.06	11	7
304373348	YP_003856557.1	E0TL76	uracil phosphoribosyltransferase	14.01	7	3
304373295	YP_003856504.1	E0TKU9	NH(3)-dependent NAD(+) synthetase	13.99	5	2
304373157	YP_003856366.1	E0TK09	Elongation factor P	13.98	3	2
304372898	YP_003856107.1	E0TKC3	Triacylglycerol lipase	13.86	14	3
304373194	YP_003856403.1	E0TK46	Probable endonuclease 4	13.82	4	2
304373442	YP_003856651.1	E0TLP2	ribonucleotide reductase stimulatory protein	13.73	3	2
304373423	YP_003856632.1	E0TLM3	hypothetical protein MHR_0644	13.70	18	6
304373198	YP_003856407.1	E0TK50	Glycyl tRNA synthetase	13.54	14	5

Table S35 (continued)

304373357	YP_003856566.1	E0TL85	Glutamyl-tRNA amidotransferase subunit A	13.54	13	6
304372915	YP_003856124.1	E0TKK7	DNA gyrase subunit A	13.45	13	8
304372916	YP_003856125.1	E0TKK8	Peptide methionine sulfoxide reductase msrA	13.33	7	2
304373358	YP_003856567.1	E0TL86	Glutamyl-tRNA amidotransferase subunit B	13.29	10	3
304373294	YP_003856503.1	E0TKU8	hypothetical protein MHR_0508	13.22	6	2
304372812	YP_003856021.1	E0TJW2	MgpA-like DHH family phosphoesterase	13.08	7	3
304373372	YP_003856581.1	E0TLA0	Glucose-6-phosphate isomerase A	12.98	17	5
304372908	YP_003856117.1	E0TKD3	Hypoxanthine phosphoribosyltransferase	12.64	6	2
304373368	YP_003856577.1	E0TL96	Neutrophil activating factor	12.50	18	2
304373144	YP_003856353.1	E0TJZ6	Protein mraZ	12.24	4	2
304372910	YP_003856119.1	E0TKK2	ABC transporter, ATP-binding protein	12.17	7	2
304373365	YP_003856574.1	E0TL93	tRNA modification GTPase mnmE	12.16	9	5
304373081	YP_003856290.1	E0TLG9	hypothetical protein MHR_0285	12.14	17	8
304373323	YP_003856532.1	E0TKX7	alanyl-tRNA synthetase	11.84	16	7
304373084	YP_003856293.1	E0TLH2	50S ribosomal protein L19	11.67	5	2
304373058	YP_003856267.1	E0TL71	Glycerophosphodiester phosphodiesterase family protein	11.62	5	2
304373249	YP_003856458.1	E0TKH7	VACB-like ribonuclease II	11.53	27	7
304373057	YP_003856266.1	E0TL70	hypothetical protein MHR_0260	11.15	18	5
304372933	YP_003856142.1	E0TKM5	Isoleucyl tRNA synthetase	11.10	19	8
304372836	YP_003856045.1	E0TK61	molecular chaperone DnaJ	11.02	5	4
304373359	YP_003856568.1	E0TL87	Putative transcriptional regulator	10.80	5	2
304373220	YP_003856429.1	E0TKE8	Expressed protein	10.64	4	3
304372907	YP_003856116.1	E0TKD2	Ribosomal RNA small subunit methyltransferase I	10.61	3	2
304372909	YP_003856118.1	E0TKD4	ABC transporter, ATP-binding protein	10.53	8	2
304373410	YP_003856619.1	E0TLD8	hypothetical protein MHR_0630	10.40	3	2
304373326	YP_003856535.1	E0TKY0	hypothetical Phosphatase yidA	10.37	3	2
304373228	YP_003856437.1	E0TKF6	prolipoprotein diacylglyceryl transferase	10.29	5	2
304373090	YP_003856299.1	E0TLH8	hypothetical protein MHR_0294	9.98	14	3
304373065	YP_003856274.1	E0TLF3	Glycosyltransferase	9.97	4	3
304373115	YP_003856324.1	E0TLK3	ABC transporter ATP-binding protein	9.97	10	3
304373152	YP_003856361.1	E0TK04	Oligopeptide transport system permease protein	9.91	5	3
304373273	YP_003856482.1	E0TKK1	ABC transporter xylose-binding lipoprotein	9.43	11	4
304372899	YP_003856108.1	E0TKC4	Lipoyltransferase and lipoate-protein ligase	9.12	6	4
304373093	YP_003856302.1	E0TLI1	COF family HAD hydrolase protein	9.09	5	2
304373367	YP_003856576.1	E0TL95	hypothetical Y+L amino acid transporter 1	8.88	7	4

Table S35 (continued)

304372932	YP_003856141.1	E0TKM4	Topoisomerase IV subunit A	8.63	13	6
304373272	YP_003856481.1	E0TKK0	DNA polymerase III alpha subunit	8.58	19	6
304373239	YP_003856448.1	E0TKG7	hypothetical protein MHR_0453	8.56	2	2
304373456	YP_003856665.1	E0TLQ6	Lipoprotein	8.37	16	4
304372873	YP_003856082.1	E0TK98	GTP-binding protein LepA	8.35	5	3
304373416	YP_003856625.1	E0TLE4	Oligopeptide transport system permease protein	8.33	10	2
304373114	YP_003856323.1	E0TLK2	ABC transporter permease protein	8.27	34	14
304373030	YP_003856239.1	E0TL43	Leucyl-trna synthetase protein	8.25	17	5
304372945	YP_003856154.1	E0TKN7	Histidyl-tRNA synthetase	8.22	7	2
304373347	YP_003856556.1	E0TL75	hypothetical protein MHR_0562	8.10	5	2
304373063	YP_003856272.1	E0TLF1	ATP-dependent helicase PcrA	8.09	10	4
304373411	YP_003856620.1	E0TLD9	Chromosome replication initiation and membrane attachment protein	7.95	10	2
304373310	YP_003856519.1	E0TKW4	Predicted GTPase, probable translation factor	7.90	4	2
304373263	YP_003856472.1	E0TKJ1	Phosphotransferase system (PTS) enzyme I	7.88	8	4
304372902	YP_003856111.1	E0TKC7	DNA polymerase III gamma and tau subunit	7.87	6	4
304373046	YP_003856255.1	E0TL59	101 kDa protein	7.76	9	5
304373009	YP_003856218.1	E0TL22	Phosphotransferase enzyme family protein	7.47	3	2
304372867	YP_003856076.1	E0TK92	ABC transporter permease protein	7.29	6	3
304372877	YP_003856086.1	E0TKA2	Amino acid or sugar ABC transport system, permease protein	7.22	9	3
304372931	YP_003856140.1	E0TKM3	Topoisomerase IV subunit B	7.09	6	3
304373054	YP_003856263.1	E0TL67	Cation-transporting ATPase family protein	6.90	4	3
304373153	YP_003856362.1	E0TK05	Oligopeptide transport system permease protein	6.86	3	2
304373076	YP_003856285.1	E0TLG4	lipoate-protein ligase A	6.65	13	3
304373218	YP_003856427.1	E0TKE6	Hexosephosphate transport protein	6.64	11	4
304372889	YP_003856098.1	E0TKB4	Heat-inducible transcription repressor hrcA	6.53	4	2
304373382	YP_003856591.1	E0TLB0	Putative ATP-binding helicase protein	6.49	12	6
304373434	YP_003856643.1	E0TLN4	hypothetical protein MHR_0655	6.32	3	3
304373044	YP_003856253.1	E0TL57	Ser/Thr protein phosphatase family protein	6.30	2	2
304373002	YP_003856211.1	E0TL15	DNA-cytosine methyltransferase family protein	6.14	4	2
304373043	YP_003856252.1	E0TL56	hypothetical protein MHR_0246	6.13	2	2
304372920	YP_003856129.1	E0TKL2	uncharactized deoxyribonuclease yabD	6.12	6	2
304373317	YP_003856526.1	E0TKX1	Lysyl-tRNA synthetase 1	5.89	7	2
304373127	YP_003856336.1	E0TLL5	methionyl tRNA synthetase	5.88	5	3
304373101	YP_003856310.1	E0TLI9	aminotransferase class V	5.74	3	2
304373277	YP_003856486.1	E0TKT1	Transcription termination-antitermination factor nusA	5.21	7	3

Table S35 (continued)

304373118	YP_003856327.1	E0TLK6	Glutamyl-tRNA synthetase	5.14	6	3
304373374	YP_003856583.1	E0TLA2	DNA polymerase III alpha subunit	4.46	16	4
304373328	YP_003856537.1	E0TKY2	Phenylalanyl-tRNA synthetase beta chain	3.98	6	2
304372814	YP_003856023.1	E0TJW4	valyl-tRNA synthetase	3.76	3	2
304373438	YP_003856647.1	E0TLN8	Lipoprotein	3.56	5	2
304373219	YP_003856428.1	E0TKE7	MG2+ ion transporter	3.46	4	2
304373309	YP_003856518.1	E0TKW3	hypothetical protein MHR_0523	3.33	5	2
304373151	YP_003856360.1	E0TK03	hypothetical protein MHR_0358	2.87	8	6
304372846	YP_003856055.1	E0TK71	101 kDa protein	2.74	2	2
304373150	YP_003856359.1	E0TK02	Lipoprotein	2.55	3	3
304373095	YP_003856304.1	E0TLI3	Putative lipoprotein	2.28	4	2
304373089	YP_003856298.1	E0TLH7	hypothetical protein MHR_0293	1.80	4	4

Table S36. Broth culture *M. hyorhinis* proteins

NCBI GI	NCBI Accession	UniProt Accession	Protein Description	% Coverage	Unique Spectra	Unique Peptides
304373345	YP_003856554.1	E0TL73	Elongation factor Tu	66.67	378	20
304372963	YP_003856172.1	E0TKQ5	hypothetical protein MHR_0162	65.77	526	23
304373302	YP_003856511.1	E0TKV6	Pyruvate dehydrogenase E1 component beta subunit	63.41	243	19
304373392	YP_003856601.1	E0TLC0	Glyceraldehyde 3-phosphate dehydrogenase C	61.86	224	17
304372893	YP_003856102.1	E0TKB8	Fructose-bisphosphate aldolase class II transmembrane protein	61.81	49	12
304373405	YP_003856614.1	E0TLD3	High affinity transport system protein p37	60.94	130	23
304373112	YP_003856321.1	E0TLK0	FMN-dependent NADH-azoreductase	60.20	224	11
304373303	YP_003856512.1	E0TKV7	Pyruvate dehydrogenase E1-alpha subunit	60.11	227	19
304373244	YP_003856453.1	E0TKH2	Pentitol phosphotransferase enzyme II, B component	60.00	17	4
304373217	YP_003856426.1	E0TKE5	L-lactate dehydrogenase	59.74	425	18
304373166	YP_003856375.1	E0TK18	DNA-directed RNA polymerase subunit alpha	59.46	85	15
304373258	YP_003856467.1	E0TKI6	Thiol-disulfide isomerase and thioredoxin	59.41	59	5
304372835	YP_003856044.1	E0TK60	aminopeptidase	57.83	106	22
304373097	YP_003856306.1	E0TLI5	Acetate kinase AckA	57.46	93	18
304373113	YP_003856322.1	E0TLK1	FMN-dependent NADH-azoreductase 1	56.19	89	8
304373187	YP_003856396.1	E0TK39	50S ribosomal protein L2	55.16	56	12
304373300	YP_003856509.1	E0TKV4	pyruvate dehydrogenase E3 component dihydrolipoamide dehydrogenase	54.53	167	26
304373393	YP_003856602.1	E0TLC1	molecular chaperone DnaK	53.43	447	28
304372926	YP_003856135.1	E0TKL8	30S ribosomal protein S9	51.52	20	4
304373016	YP_003856225.1	E0TL29	ATP synthase C chain, sodium ion specific lipid-binding protein	51.38	23	3
304373255	YP_003856464.1	E0TKI3	Enolase	49.56	167	17
304373096	YP_003856305.1	E0TLI4	Phosphate acetyltransferase	49.06	47	11
304372925	YP_003856134.1	E0TKL7	50S ribosomal protein L13	48.97	19	5
304373314	YP_003856523.1	E0TKW8	adenine phosphoribosyltransferase	48.04	25	6
304373287	YP_003856496.1	E0TKU1	30S ribosomal protein S7	46.79	15	6
304372890	YP_003856099.1	E0TKB5	Heat shock protein	46.54	18	10
304373264	YP_003856473.1	E0TKJ2	50S ribosomal protein L21	45.45	9	5
304373068	YP_003856277.1	E0TLF6	50S ribosomal protein L1	45.22	28	7
304373390	YP_003856599.1	E0TLB8	Trigger factor	45.17	166	21
304373351	YP_003856560.1	E0TL79	Probable purine nucleoside phosphorylase transmembrane protein	44.40	39	9
304373349	YP_003856558.1	E0TL77	Deoxyribose-phosphate aldolase 1	44.39	26	6

Table S36 (continued)

304372957	YP_003856166.1	E0TKP9	Triosephosphate isomerase	43.21	22	7
304373174	YP_003856383.1	E0TK26	50S ribosomal protein L18	43.09	45	5
304373179	YP_003856388.1	E0TK31	50S ribosomal protein L24	42.99	7	3
304373084	YP_003856293.1	E0TLH2	50S ribosomal protein L19	41.67	9	6
304373292	YP_003856501.1	E0TKU6	Pyruvate kinase	40.63	74	16
304373409	YP_003856618.1	E0TLD7	membrane protease subunits, stomatin/prohibitin-like protein, partial	40.00	13	5
304373185	YP_003856394.1	E0TK37	50S ribosomal protein L22	39.67	25	5
304373073	YP_003856282.1	E0TLG1	Lipoprotein	39.52	64	17
304373176	YP_003856385.1	E0TK28	30S ribosomal protein S8	39.23	11	4
304373175	YP_003856384.1	E0TK27	50S ribosomal protein L6	39.11	43	5
304373021	YP_003856230.1	E0TL34	Proton-translocating ATPase, beta subunit	38.72	54	12
304372964	YP_003856173.1	E0TKQ6	xylose ABC transporter ATP-binding protein	38.58	68	17
304372872	YP_003856081.1	E0TK97	Histone-like DNA-binding protein	37.78	3	2
304373312	YP_003856521.1	E0TKW6	Single-stranded DNA-binding protein	37.43	11	5
304373108	YP_003856317.1	E0TLJ6	Methyltransferase type 11	36.72	12	4
304373003	YP_003856212.1	E0TL16	aminopeptidase	36.01	58	12
304373041	YP_003856250.1	E0TL54	Transcription antitermination protein	35.86	18	5
304373335	YP_003856544.1	E0TKY9	ATP-dependent serine proteinase, heat shock protein	35.52	88	20
304373376	YP_003856585.1	E0TLA4	Ribosome recycling factor	35.48	14	4
304373222	YP_003856431.1	E0TKF0	Phosphoglycerate kinase	34.58	60	11
304373246	YP_003856455.1	E0TKH4	hypothetical protein MHR_0460	34.00	24	8
304373227	YP_003856436.1	E0TKF5	Thioredoxin reductase	33.11	35	10
304373301	YP_003856510.1	E0TKV5	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	33.01	41	10
304373178	YP_003856387.1	E0TK30	50S ribosomal protein L5	32.60	27	4
304373308	YP_003856517.1	E0TKW2	Thiol peroxidase	31.98	26	4
304373167	YP_003856376.1	E0TK19	30S ribosomal protein S13	31.97	7	3
304373182	YP_003856391.1	E0TK34	50S ribosomal protein L29	31.88	5	3
304373200	YP_003856409.1	E0TK52	Elongation factor Ts	31.08	40	8
304373032	YP_003856241.1	E0TL45	50S ribosomal protein L9	31.03	9	3
304373146	YP_003856355.1	E0TJZ8	cell division protein FtsZ	31.02	12	8
304373259	YP_003856468.1	E0TKI7	prolyl-tRNA synthetase	30.83	35	10
304373201	YP_003856410.1	E0TK53	30S ribosomal protein S2	30.54	31	7
304373288	YP_003856497.1	E0TKU2	translation elongation factor G	30.52	52	15
304372840	YP_003856049.1	E0TK65	NADH oxidase	30.35	146	12

Table S36 (continued)

304372857	YP_003856066.1	E0TK82	50S ribosomal protein L7/L12	30.08	22	4
304373165	YP_003856374.1	E0TK17	50S ribosomal protein L17	30.00	10	4
304373181	YP_003856390.1	E0TK33	30S ribosomal protein S17	30.00	12	3
304373370	YP_003856579.1	E0TL98	LemA-family protein	30.00	13	4
304373184	YP_003856393.1	E0TK36	30S ribosomal protein S3	29.22	19	7
304373432	YP_003856641.1	E0TLN2	ATP synthase subunit B	29.09	22	8
304373149	YP_003856358.1	E0TK01	Oligoendopeptidase F	28.74	51	14
304373186	YP_003856395.1	E0TK38	30S ribosomal protein S19	28.57	10	2
304372862	YP_003856071.1	E0TK87	hypothetical protein MHR_0059	28.52	13	4
304373158	YP_003856367.1	E0TK10	Probable transketolase transmembrane protein	28.46	48	14
304373348	YP_003856557.1	E0TL76	uracil phosphoribosyltransferase	28.02	15	6
304373305	YP_003856514.1	E0TKV9	50S ribosomal protein L20	27.73	6	3
304373428	YP_003856637.1	E0TLM8	TrkA-C domain protein	27.56	7	4
304372949	YP_003856158.1	E0TKP1	Xaa-pro aminopeptidase	26.91	16	6
365897170	YP_004956716.1	E4PYU7	transcription elongation factor	26.83	8	4
304372865	YP_003856074.1	E0TK90	uncharactized lipoprotein	26.69	30	14
304373189	YP_003856398.1	E0TK41	50S ribosomal protein L4	26.44	21	6
304373191	YP_003856400.1	E0TK43	30S ribosomal protein S10	25.96	8	2
304373023	YP_003856232.1	E0TL36	101 kDa protein	25.87	59	19
304373350	YP_003856559.1	E0TL78	Thymidine phosphorylase	25.29	19	9
304373190	YP_003856399.1	E0TK42	50S ribosomal protein L3	25.11	14	6
304373010	YP_003856219.1	E0TL23	GTPase obg	24.88	20	8
304373275	YP_003856484.1	E0TKS9	Translation initiation factor IF-2	24.83	25	10
304373372	YP_003856581.1	E0TLA0	Glucose-6-phosphate isomerase A	24.68	27	8
304373086	YP_003856295.1	E0TLH4	30S ribosomal protein S16	24.44	4	2
304373188	YP_003856397.1	E0TK40	50S ribosomal protein L23	24.39	29	4
304373418	YP_003856627.1	E0TLE6	Lipoprotein	23.57	65	17
304373240	YP_003856449.1	E0TKG8	Sugar isomerase SgaE	22.95	17	4
304373410	YP_003856619.1	E0TLD8	hypothetical protein MHR_0630	22.54	4	3
304373286	YP_003856495.1	E0TKU0	30S ribosomal protein S12	22.30	6	3
304372859	YP_003856068.1	E0TK84	DNA-directed RNA polymerase subunit beta'	22.25	49	20
304373105	YP_003856314.1	E0TLJ3	Phosphopentomutase	22.22	17	5
304373093	YP_003856302.1	E0TLI1	COF family HAD hydrolase protein	22.08	13	5
304373318	YP_003856527.1	E0TKX2	cell division protease FtsH	22.04	37	9
304373129	YP_003856338.1	E0TLL7	Nicotinate phosphoribosyltransferase	21.79	11	5

Table S36 (continued)

304373425	YP_003856634.1	E0TLM5	Pyrophosphatephospho hydrolase	21.74	19	3
304373144	YP_003856353.1	E0TJZ6	Protein mraZ	21.09	4	3
304373242	YP_003856451.1	E0TKH0	hexulose 6 phosphate synthase	20.98	7	4
304373173	YP_003856382.1	E0TK25	30S ribosomal protein S5	20.63	15	5
304373183	YP_003856392.1	E0TK35	50S ribosomal protein L16	20.44	11	2
304373332	YP_003856541.1	E0TKY6	6-phosphofructokinase	20.43	33	6
304372864	YP_003856073.1	E0TK89	uncharactized lipoprotein	20.36	33	11
304373198	YP_003856407.1	E0TK50	Glycyl tRNA synthetase	20.31	29	8
304373019	YP_003856228.1	E0TL32	ATP synthase subunit alpha	20.28	21	9
304373368	YP_003856577.1	E0TL96	Neutrophil activating factor	20.14	7	2
304373284	YP_003856493.1	E0TKT8	Protein P115	20.12	36	15
304373006	YP_003856215.1	E0TL19	5'-nucleotidase	20.10	13	5
304373147	YP_003856356.1	E0TJZ9	hypothetical protein MHR_0354	19.90	11	5
304373194	YP_003856403.1	E0TK46	Probable endonuclease 4	19.64	9	3
304373438	YP_003856647.1	E0TLN8	Lipoprotein	19.24	35	12
304373081	YP_003856290.1	E0TLG9	hypothetical protein MHR_0285	19.22	35	13
304373169	YP_003856378.1	E0TK21	Methionine aminopeptidase	18.73	4	3
304373243	YP_003856452.1	E0TKH1	Pentitol phosphotransferase enzyme II, A component	18.59	8	2
304373408	YP_003856617.1	E0TLD6	hypothetical protein MHR_0628	18.14	25	11
304373313	YP_003856522.1	E0TKW7	30S ribosomal protein S6	18.13	4	3
304373013	YP_003856222.1	E0TL26	putative acyl carrier protein	18.06	5	2
304372959	YP_003856168.1	E0TKQ1	Serine hydroxymethyltransferase 3	17.94	17	4
304373156	YP_003856365.1	E0TK08	Oligoendopeptidase F	17.60	20	7
304373249	YP_003856458.1	E0TKH7	VACB-like ribonuclease II	17.58	19	9
304373172	YP_003856381.1	E0TK24	50S ribosomal protein L15	17.57	14	3
304372813	YP_003856022.1	E0TJW3	dihydrolipoamide dehydrogenase	17.07	21	7
304373380	YP_003856589.1	E0TLA8	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	16.96	22	8
304372852	YP_003856061.1	E0TK77	5'-nucleotidase	16.74	18	8
304373416	YP_003856625.1	E0TLE4	Oligopeptide transport system permease protein	16.41	8	4
304373061	YP_003856270.1	E0TLE9	Fatty acid-binding protein DegV-like protein	16.26	4	3
304373281	YP_003856490.1	E0TKT5	Predicted kinase, related to dihydroxyacetone kinase	16.18	23	6
304372886	YP_003856095.1	E0TKB1	30S ribosomal protein S4	16.10	11	4
304373082	YP_003856291.1	E0TLH0	hypothetical protein MHR_0286	16.05	5	4
304373029	YP_003856238.1	E0TL42	Fructose permease IIC component	15.90	21	8
304373407	YP_003856616.1	E0TLD5	P60-like lipoprotein	15.57	15	6

Table S36 (continued)

304373046	YP_003856255.1	E0TL59	101 kDa protein	15.52	31	12
304372996	YP_003856205.1	E0TL09	Lipoprotein	15.40	23	10
304373283	YP_003856492.1	E0TKT7	Ribonuclease III	15.11	3	2
304373152	YP_003856361.1	E0TK04	Oligopeptide transport system permease protein	15.09	11	5
304373151	YP_003856360.1	E0TK03	hypothetical protein MHR_0358	14.85	73	29
304373456	YP_003856665.1	E0TLQ6	Lipoprotein	14.61	18	8
304373310	YP_003856519.1	E0TKW4	Predicted GTPase, probable translation factor	14.44	9	4
304373030	YP_003856239.1	E0TL43	Leucyl-trna synthetase protein	14.21	18	8
304373433	YP_003856642.1	E0TLN3	ATP synthase subunit A	14.20	16	6
304373221	YP_003856430.1	E0TKE9	P3	13.89	35	7
304373199	YP_003856408.1	E0TK51	Signal recognition particle protein	13.71	13	4
304372858	YP_003856067.1	E0TK83	DNA-directed RNA polymerase subunit beta	13.67	24	15
304373017	YP_003856226.1	E0TL30	ATP synthase subunit b	13.23	5	2
304372812	YP_003856021.1	E0TJW2	MgpA-like DHH family phosphoesterase	13.08	8	3
304373116	YP_003856325.1	E0TLK4	Peptide chain release factor 1	13.02	8	3
304373326	YP_003856535.1	E0TKY0	hypothetical Phosphatase yidA	12.96	6	2
304373206	YP_003856415.1	E0TK58	Segregation and condensation protein B	12.81	2	2
304372952	YP_003856161.1	E0TKP4	Seryl-trna synthetase protein	12.80	12	3
304373170	YP_003856379.1	E0TK22	adenylate kinase	12.61	3	2
304372946	YP_003856155.1	E0TKN8	aspartyl tRNA synthetase	12.57	12	6
304373204	YP_003856413.1	E0TK56	Probable 1-acyl-sn-glycerol-3-phosphate acyltransferase	12.45	6	2
304373414	YP_003856623.1	E0TLE2	Oligopeptide transport system permease protein	12.39	13	8
304372933	YP_003856142.1	E0TKM5	Isoleucyl tRNA synthetase	12.22	16	9
304373180	YP_003856389.1	E0TK32	50S ribosomal protein L14	12.20	3	2
304372910	YP_003856119.1	E0TKK2	ABC transporter, ATP-binding protein	12.17	5	2
304373056	YP_003856265.1	E0TL69	Glycerol-3-phosphate dehydrogenase, putative	11.95	7	3
304373439	YP_003856648.1	E0TLN9	hypothetical protein MHR_0660	11.87	17	6
304373423	YP_003856632.1	E0TLM3	hypothetical protein MHR_0644	11.75	7	3
304373385	YP_003856594.1	E0TLB3	Threonyl-tRNA synthetase	11.70	14	7
304372879	YP_003856088.1	E0TKA4	Ag 243-5 protein	11.67	17	4
304372945	YP_003856154.1	E0TKN7	Histidyl-tRNA synthetase	11.64	9	3
304372915	YP_003856124.1	E0TKK7	DNA gyrase subunit A	11.63	13	7
304373226	YP_003856435.1	E0TKF4	Outer membrane protein-P95	11.46	19	7
304373294	YP_003856503.1	E0TKU8	hypothetical protein MHR_0508	11.16	6	2
304373295	YP_003856504.1	E0TKU9	NH(3)-dependent NAD(+) synthetase	11.11	5	2

Table S36 (continued)

304373124	YP_003856333.1	E0TLL2	Expressed protein	10.91	3	2
304373211	YP_003856420.1	E0TKD9	protein phosphatase 2C	10.89	5	2
304373360	YP_003856569.1	E0TL88	Glucosamine-6-phosphate isomerase 1	10.87	5	2
304373110	YP_003856319.1	E0TLJ8	hypothetical protein MHR_0314	10.86	6	2
304373391	YP_003856600.1	E0TLB9	hypothetical 33 kDa chaperonin	10.58	9	3
304372898	YP_003856107.1	E0TKC3	Triacylglycerol lipase	10.49	4	2
304373458	YP_003856667.1	E0TLQ8	Membrane protein oxaA	10.46	14	5
304373241	YP_003856450.1	E0TKG9	Probable L-ribulose-5-phosphate 3-epimerase ulaE	10.44	9	2
304373323	YP_003856532.1	E0TKX7	alanyl-tRNA synthetase	10.26	13	8
304373417	YP_003856626.1	E0TLE5	Oligopeptide ABC transporter permease protein	10.26	3	3
304373212	YP_003856421.1	E0TKE0	guanylate kinase	9.90	2	2
304373225	YP_003856434.1	E0TKF3	hypothetical protein MHR_0439	9.85	10	8
304373365	YP_003856574.1	E0TL93	tRNA modification GTPase mnmE	9.68	9	4
304373115	YP_003856324.1	E0TLK3	ABC transporter ATP-binding protein	9.65	5	3
304373209	YP_003856418.1	E0TKD7	Ribulose-phosphate 3-epimerase	9.63	9	2
304373346	YP_003856555.1	E0TL74	ATP-dependent protease La	9.62	15	7
304373415	YP_003856624.1	E0TLE3	Oligopeptide ABC transporter ATP binding prote	9.60	13	3
304373320	YP_003856529.1	E0TKX4	Peptidyl-tRNA hydrolase	9.42	3	2
304373358	YP_003856567.1	E0TL86	Glutamyl-tRNA amidotransferase subunit B	9.28	4	2
304373076	YP_003856285.1	E0TLG4	lipoate-protein ligase A	9.25	17	4
304373090	YP_003856299.1	E0TLH8	hypothetical protein MHR_0294	9.16	6	3
304373101	YP_003856310.1	E0TLI9	aminotransferase class V	9.14	7	3
304373048	YP_003856257.1	E0TL61	hypothetical protein MHR_0251	9.07	4	2
304372968	YP_003856177.1	E0TKR0	hypothetical protein MHR_0167	8.89	8	3
304373228	YP_003856437.1	E0TKF6	prolipoprotein diacylglyceryl transferase	8.82	3	2
304373069	YP_003856278.1	E0TLF7	CTP synthase	8.81	12	3
304373277	YP_003856486.1	E0TKT1	Transcription termination-antitermination factor nusA	8.80	3	3
304373263	YP_003856472.1	E0TKJ1	Phosphotransferase system (PTS) enzyme I	8.58	12	4
304373366	YP_003856575.1	E0TL94	ABC transporter ATP-binding protein	8.55	10	4
304373114	YP_003856323.1	E0TLK2	ABC transporter permease protein	8.35	30	15
304373218	YP_003856427.1	E0TKE6	Hexosephosphate transport protein	7.88	12	4
304373338	YP_003856547.1	E0TKZ2	Zinc metalloproteinase C	7.78	9	7
304372876	YP_003856085.1	E0TKA1	P59-like protein	7.62	4	2
304373431	YP_003856640.1	E0TLN1	hypothetical protein MHR_0652	7.56	11	2
304373057	YP_003856266.1	E0TL70	hypothetical protein MHR_0260	7.54	5	3

Table S36 (continued)

304373045	YP_003856254.1	E0TL58	DNA gyrase subunit B	7.34	7	5
304373273	YP_003856482.1	E0TKK1	ABC transporter xylose-binding lipoprotein	7.24	8	2
304372899	YP_003856108.1	E0TKC4	Lipoyltransferase and lipoate-protein ligase	6.99	6	2
304373282	YP_003856491.1	E0TKT6	Phosphate acyltransferase	6.95	5	3
304373043	YP_003856252.1	E0TL56	hypothetical protein MHR_0246	6.93	3	2
304372873	YP_003856082.1	E0TK98	GTP-binding protein LepA	6.84	3	2
304372918	YP_003856127.1	E0TKL0	Ribose-phosphate pyrophosphokinase	6.75	2	2
304373150	YP_003856359.1	E0TK02	Lipoprotein	6.66	7	4
304373331	YP_003856540.1	E0TKY5	hypothetical protein MHR_0546	6.63	11	3
304373065	YP_003856274.1	E0TLF3	Glycosyltransferase	6.34	3	2
304373229	YP_003856438.1	E0TKF7	Excinuclease ATPase subunit-like protein	6.28	15	7
304373153	YP_003856362.1	E0TK05	Oligopeptide transport system permease protein	6.24	4	2
304373317	YP_003856526.1	E0TKX1	Lysyl-tRNA synthetase 1	6.10	8	4
304373352	YP_003856561.1	E0TL80	Protein translocase subunit secA	6.10	6	5
304373230	YP_003856439.1	E0TKF8	hypothetical protein MHR_0444	5.75	4	3
304372889	YP_003856098.1	E0TKB4	Heat-inducible transcription repressor hrcA	5.40	2	2
304372836	YP_003856045.1	E0TK61	molecular chaperone DnaJ	5.38	4	2
304373066	YP_003856275.1	E0TLF4	Putative asparaginyl-trna synthetase protein	5.38	6	2
304372807	YP_003856016.1	E0TJV7	DNA polymerase III beta subunit	5.35	4	2
304373037	YP_003856246.1	E0TL50	Cysteinyl-tRNA synthetase	5.25	2	2
304372965	YP_003856174.1	E0TKQ7	Xylose ABC transporter permease protein	5.24	7	2
304373089	YP_003856298.1	E0TLH7	hypothetical protein MHR_0293	5.01	16	7
304373063	YP_003856272.1	E0TLF1	ATP-dependent helicase PcrA	4.91	4	3
304373404	YP_003856613.1	E0TLD2	Probable ABC transporter ATP-binding protein p29	4.76	2	2
304373357	YP_003856566.1	E0TL85	Glutamyl-tRNA amidotransferase subunit A	4.37	6	2
304373382	YP_003856591.1	E0TLB0	Putative ATP-binding helicase protein	4.10	6	4
304373443	YP_003856652.1	E0TLP3	ribonucleotide-diphosphate reductase subunit alpha	3.88	11	2
304373334	YP_003856543.1	E0TKY8	Membrane nuclease, lipoprotein	3.82	2	2
304372845	YP_003856054.1	E0TK70	hypothetical protein MHR_0042	3.55	3	2

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-question everything

-never stop learning

-keep dancing, even when the music stops

…you, my darlings, make my world go round!